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Effects of Caesalpinia sappan components in human arthritic chondrocytes and synoviocytes

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Athena Su Agin

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Table of Contents

Abstract

Caesalpinia sappan is a demotic medical plant in southeast Asia, where it is an inherent part of the traditional Chinese medicine. The extracts of the heartwood are known to be anti-inflammatory, analgesic and blood circulation improving.

In this thesis, I aimed to determine the impact of the plant components on human chondrocytes and synoviocytes. In this working process, I extracted the main five components of Caesalpinia sappan using preparative high performance liquid chromatography. The resulting components were: episappanol, protosappanin C, brazilin, protosappanin B and sappanol. In order to work with non-toxic concentrations of each component, non-radioactive cell proliferation and cytotoxicity assays were performed. The cells were treated with each component in presence or absence of IL-1β and gene expression levels of IL1B, MMP13 and HMOX1 as well as protein levels of HMOX-1 were analyzed using real time quantitative polymerase chain reaction and Western blotting, respectively.

We found that brazilin had a strong influence on gene expression levels, whereas the other components showed no effect. Of note, brazilin strongly suppressed an IL-1β-mediated increase of IL1B and MMP13 in osteoarthritic chondrocytes. This is in agreement with the induction of gene and protein levels of HMOX-1 by brazilin treatment. Interestingly, osteoarthritic synoviocytes showed no response to brazilin treatment except of one patient-derived cell population. For this cell population, it was observed that brazilin significantly inhibited IL-1β-mediated up-regulation of IL1B and MMP13. In agreement, brazilin also led to a significant increase of HMOX1 gene expression levels in this cell population. These results might be related to the different systemic inflammation status of this patient.

The impact of brazilin on gene expression levels was also determined in rheumatoid arthritic synoviocytes. The IL-1β-mediated increase of IL1B and MMP13 mRNA levels was suppressed whereas HMOX1 mRNA levels were significantly up-regulated by brazilin. In non-osteoarthritic chondrocytes, brazilin significantly raised HMOX1 gene expression rates in presence or absence of IL-1β. However, brazilin did not induce any effect on IL1B mRNA levels and just a slight suppression of IL-1β-mediated MMP13 up-regulation in non-osteoarthritic chondrocytes.

In summary, this thesis provides first evidence that the beneficial effects of Caesalpinia sappan extracts in the context of the arthritic disease can be attributed to the activity of brazilin. The results further indicate that the anti-inflammatory as well as the anti-matrix-degenerative impact of brazilin correlates with an increase of HMOX1 mRNA levels induced by brazilin.

Zusammenfassung

Caesalpinia sappan ist eine Heilpflanze der Volksmedizin Südostasiens, wo sie ein fester Bestandteil der traditionellen chinesischen Medizin ist. Die Extrakte des Kernholzes sind bekannt für ihre entzündungshemmende, schmerzlindernde und Blutzirkulation-verbessernde Wirkung.

Im Rahmen dieser Diplomarbeit wurde versucht, die Wirkung der pflanzlichen Komponenten auf humane Chondrozyten und Synoviozyten zu erforschen. Dafür wurden Genexpressionsraten von IL1B, MMP13 und HMOX1, sowie auch Proteinexpressionsraten von HMOX-1 analysiert.

Innerhalb des Arbeitsprozesses wurden die fünf Hauptkomponenten von Caesalpinia sappan mittels Hochleistungsflüssigkeitschromatographie extrahiert. Die resultierenden Komponenten waren: Episappanol, Protosappanin C, Brazilin, Protosappanin B und Sappanol. Des weitern wurden nicht-radioaktive Zell-Proliferation und -Toxizität Tests durchgeführt um nicht-toxische Konzentrationen für alle fünf pflanzlichen Komponenten zu ermitteln. Die Zellen wurden mit IL-1β stimuliert und mit nicht-toxischen Konzentrationen der Komponenten behandelt. Resultierende Genexpressionsraten wurden mittels quantitativer Echtzeit-Polymerase-Kettenreaktion quantifiziert.

Brazilin zeigte einen starken Einfluss auf mRNA Level, während die restlichen Komponenten keinen Effekt aufwiesen. Es konnte festgestellt werden, dass Brazilin in arthritischen Chondrozyten eine IL-1β-induzierte Erhöhung von IL1B und MMP13 hemmt. Übereinstimmend dazu konnte Brazilin die mRNA Level von HMOX1 und auch Protein Level von HMOX-1 erhöhen, wobei die Proteinexpressionsraten mittels Western blot ermittelt wurden. Interessanterweise haben arthritische Synoviozyten keinerlei Reaktionen auf eine Behandlung mit Brazilin gezeigt, mit Ausnahme der Zellen eines einzelnen Patienten. Nur für diesen Patienten war eine Brazilin Wirkung erkennbar, wobei es zu einer signifikanten Inhibierung von IL-1β-induzierter Überexpression von IL1B und MMP13 kam. In Übereinstimmung dazu hat Brazilin ebenfalls signifikant HMOX1 Genexpressionslevel erhöht. Diese Resultate könnten möglicherweise mit einem veränderten Entzündungsstatus dieses Patienten in Zusammenhang stehen.

Die Wirkung von Brazilin auf Genexpressionslevel war auch in rheumatoid arthritischen Synoviozyten ersichtlich. Der IL-1β-induzierte Anstieg von IL1B und MMP13 wurde

gehemmt und Brazilin hat HMOX1 mRNA Level signifikant angehoben. In nicht arthritischen Chondrozyten konnte Brazilin die HMOX1 Genexpressionsraten ebenfalls signifikant anheben, jeweils in Ab- und Anwesenheit von IL-1β. In nicht arthritischen Chondrozyten zeigte Brazilin dagegen keinen Effekt auf IL1B mRNA Level und führte nur zu einer leichten Senkung von MMP13 Genexpressionsraten.

Zusammengefasst zeigen die Resultate dieser Diplomarbeit, dass die Wirkungen von Caesalpinia sappan Extrakten in gelenksassoziierierten humanen Zellen auf die Aktivität des Brazilin zurückzuführen sind. Außerdem zeigte sich, dass die Wirkung von Brazilin mit einer Anhebung von HMOX1 Genexpressionsraten korreliert.

1. Introduction

1.1. The knee joint

The biggest and the most complex joint of the human body is the knee joint, as seen in Figure 1. 1.. Because it is loaded with the whole body weight, the knee joint has to be particularly resistant to mechanical stress.

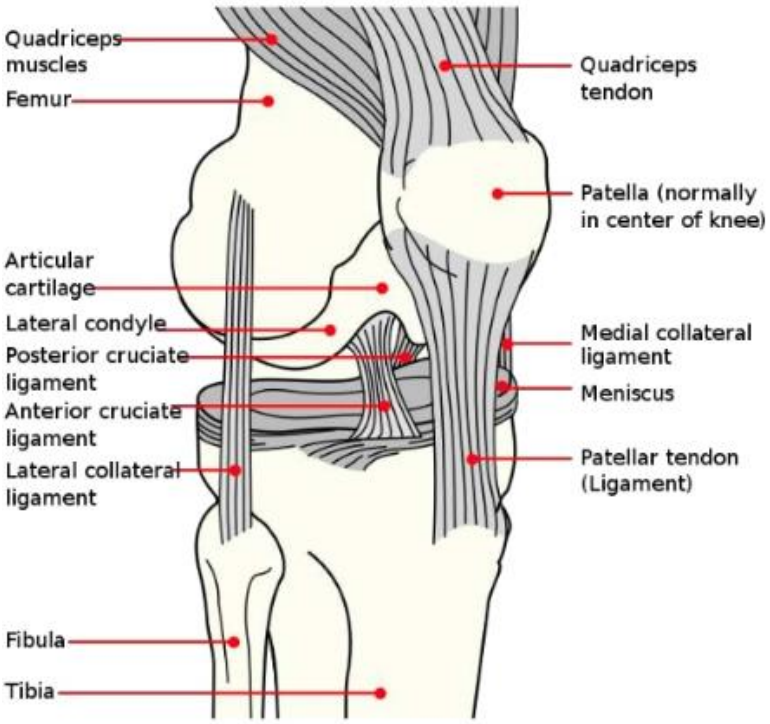

Figure 1. 1. Schematically view of a human knee joint

(Image taken from http://www.arthroseorthopaedie.de/images/knieanatomie_1_l.gif)

Inside the knee joint, three articulating bones encounter, the thighbone (femur), the shinbone (tibia) and the kneecap (patella). As the bones alone would compose an instable construct, they are stabilized in the space of the joint capsule of the knee. The strength and stability of the joint capsule is provided by ligaments, which are strapping the inside and outside of the joint. These ligaments include collateral ligaments (medialis and lateralis), but also cruciate ligaments, which are cutting across each other inside, in the middle of the joint. The posterior and the anterior cruciate ligament are connecting the femur with the tibia. The patellar tendon, also a ligament, is connecting the patella with the tibia. These ligaments are the flexible parts of the muscles and are allowing a connection between the muscle and the bone. The patella is connected to the femoral muscle by the quadriceps tendon.

Between the joint capsule and the joint cavity is the synovial membrane. This membrane is producing the synovial fluid that lubricates the joint surface and reduces in this way the friction within the join to a minimum. Because the cartilage is avascular the synovial fluid also adopted the nutrient supplying function. To strengthen articular cartilage, physical activity is necessary, as it improves nutrient absorption.

However, the joint fluid is not only located in the cavity of the joint, but also in synovial bursae. The bursae are pouch formed parts of the synovial membrane, filled with synovial liquid, that are located in spots of the knee where muscle, skin or tendon are rubbing against the bone, to absorb high loads.

As already mentioned before, the articular cartilage prohibits that bones rub against each other and is able to transfer immense power in this process. In addition, the two menisci serve as cushions between the tibia and the femur. They are wedge-shaped half rings that are attached on their peripheral borders to the joint capsule.

Movements of the knee joint are possible as flexion, which is carried out mainly by musculus biceps femoris and semitendinosus, as extension, carried out mainly by musculus quadriceps femoris, as medial rotation, mainly carried out by the politeus muscle and lateral rotation done only by musculus biceps femoris.

Injuries of the knee joint are very common, because it is a weight-bearing, mobile, low located joint that is necessary for everyday activities, as well as for athletic activities. It is prone for acute injuries, as well as for chronic diseases such as osteoarthritis (Schünke et al., 2014).

1.1.1. Functional structure and composition of articular cartilage

Cartilage-tissue consists of chondrocytes and extracellular matrix, whose most important component is proteoglycans and collagen fibrils. The hyaline cartilage with its mesenchymal origin shows a very complex tissue organization, it covers the surface of the part of the bone, which is an integral part of the joint. It is deformable but returns immediately in its original shape as soon the load lowers. Consequently, the articular cartilage acts as an absorber in the musculoskeletal system. Despite its extraordinary mechanical benefits, it lacks of metabolic activity and especially of regenerative efficacy (Brix et al., 2012).

The characteristic properties of the articular cartilage are explainable by its organization. The main components are water, collagen and proteoglycan. In contrast, cells make up only 5% of the wet weight (Pearl et al., 2005) but still the chondrocyte metabolism determines the extracellular matrix. And a stable equilibrium state of the matrix is required for the homeostasis of the joint tissue. Because the joint is hypocellular and contains no vasculature, no nervous tissue as well as no lymph, it is dependent on the synovia and on the subchondral bone (Madry et al., 2010) to receive by diffusion oxygen as well as essential nutrients by diffusion.

1.1.2. Function and structure of the synovia

Healthy synovia, with its deformable and ductile features, gives adjoining tissue without this feature the possibility to move. In this process, synovia maintains the movement as it compensates flexion, extension, medial rotation and lateral rotation during knee motion. Also, it is responsible for an adequate production of synovial liquid, which covers the joint surface as lubricating film. Therefore, glycoproteins are essential which occur in the surface of the cartilage and the synovia, glycoproteins called lubricin or superficial zone protein (Smith, 2011). The synovia is also responsible for the consistency of chondrocytes and their nutritive supplementation.

Regarding the vascular net, capillaries are found just beneath or in the intima, the outer layer of the synovia. These are providing not only a nutritive supply for the synovial tissue itself, but also for the avascular cartilage. Cells of the synovial intima can be of two different types, fibroblasts or macrophages, although they can both not be set coequal to similar cells in other tissues. Synovial fibroblasts produce hyaluronan, a long chain polymer that traps water in the cavity of the joint and gives the synovial fluid its ductile character together with the glycoprotein lubricin. Synovial macrophages, in contrast, are responsible for phagocytosis of unrequested substances in the joint fluid (Wechalecker et al., 2014).

The production of inflammatory cytokines including interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α), as well as extra cellular matrix-degenerating, matrix metalloproteinase 13 (MMP-13), are observable also in healthy synovial tissue. In contrast to patients with osteoarthritis (Benito et al., 2014) or rheumatoid arthritis (RA), however, these production rates are far lower.

1.2. Osteoarthritis

Osteoarthritis (OA) is a degenerative, chronic disease of the joint and the most common form of arthritis. It can occur in any joint, however, the knee, the hips, the hands and the spine are the most commonly affected joints. Because of its accompaniment with the structural and functional degeneration of the joint, this chronic complaint can be seen as the main cause for disability and dysfunction in elderly people (Guccione et al., 1994).

A confirmed diagnosis is only possible in end-stage disease. At this point of time, clear symptoms are observable like joint pain, stiffness, dysfunction and deformation, as well as radiographic attributes considering joint space and the structure and composition of the joint. In contrast, early staged OA appears with very elusive symptoms or remains asymptomatic (Guccione et al., 1994). Since the articular cartilage is an essential part of the musculoskeletal system, an arthritic process in this area can lead in many cases to a joint replacement, as it occurs often in the biggest joint of the human body, the knee joint.

Therefore, it is not only necessary to set the focus on new innovative strategies of diagnostics in this field but also on novel therapeutic strategies, which can not only delay the progression of disease but also improve the degenerative status.

1.2.1. Pathogenesis of OA

In OA, the impaired function of chondrocytes and synoviocytes is attributed to, inter alia, pro-inflammatory cytokines such as IL-1β and TNF-α, which are additionally leading to higher levels of MMP-13 (Skolove et al., 2013). The higher concentration of these three substances in chondrocytes, as well as in synoviocytes, can lead to a disturbed equilibrium of the anabolic and catabolic system of the cartilage, as shown in Figure 1. 2..

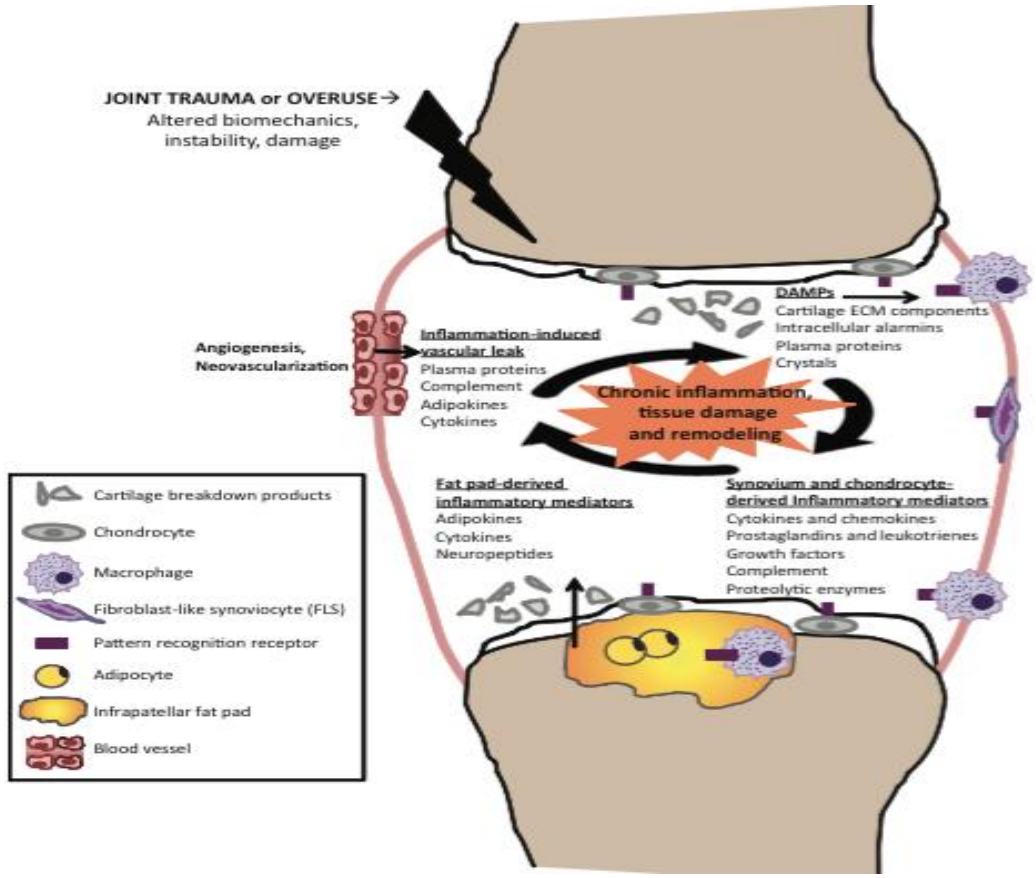

Figure 1. 2. Chronic inflammation of the joint as an initiator for OA

Tissue damage, as a result to joint trauma or overuse, leads to the production of damage-associated molecular patterns (DAMPs), involving degeneration products of the extracellular matrix (ECM) within the cartilage and intracellular alarmist, that are activating through receptor docking macrophages and fibroblasts of the synovia, as well as chondrocytes, that are producing consequently cytokines. This inflammation process causes angiogenesis and raised permeability of vasculature. Now plasma proteins enter in the joint and act as DAMPs. This whole process results in a vicious circle in which cytokines provide cartilage degeneration and the degeneration inflammatory mediators as proteolytic enzymes (Skolove et al., 2013).

Causal OA can occur because of an overuse, like it happens to be the case in obesity, or in consequence of a joint trauma or innate reasons like malposition of the joint. All these causes lead to a chronic inflammation, which conveys OA.

Symptomatic evidence for OA is evolving slowly and malicious. It is often recognized in a late state of the disease. The main symptoms are pain that is possible to occur during or after a movement, swelling as well as stiffness of the joint, which are most likely to occur after a period of physical inactivity like in the mornings, bone spurs, an exostosis of the bone feeling like hard little balls under the skin formed around the arthritic joint and consequently tenderness, because of less weight carrying capacity of the joint and a loss of flexibility.

1.2.2. Treatment of OA

For OA, there is no treatment that is able to cure the disease, but many predisposing factors might be targeted by changes in lifestyle. Regarding obese patients a reduction of body weight might drastically increase life quality (Glyn-Jones et al., 2015).

On the other hand, there is also the possibility of surgical intervention, as final option if conservative treatment is not successful. In the process of joint replacement, degenerated joint surfaces can be replaced with metal or plastic implants. This intervention can also affect the whole joint within a total joint replacement.

The conservative therapy strategy does not only consider lifestyle changes including physical activity and altered eating habits, but also medical intervention. The focus is set on reducing symptoms by the use of analgesics in which acetaminophen (paracetamol) and non-steroidal anti-inflammatory drugs (NSAIDs) are playing a key role. Paracetamol is able to achieve pain reduction but not a decrease of inflammation levels. Dosing recommendations should be followed as higher dosages can lead to damages of the liver. Contrary, NSAIDs are able to reduce inflammation as well as symptomatic pain, but they can lead to stomach bleeding and cardiovascular problems, which is the reason why they are not recommended for patients over an age of 65. A topical application also leads to relevant plasma rates of NSAIDs with the advantage of lower rates of unwanted side effects.

In summary, following major clinical guidelines, if paracetamol and the topical application of NSAIDs are not effectively in pain relief, oral NSAIDs are prescribed, which have to be unconditionally combined in long term use with gastric protecting medicaments like proton pump inhibitors. If still NSAIDs are not able to reduce the pain sufficiently, a short time use of opioid analgesics is allowed (Aktories et al., 2009).

1.3. RA and its pathogenesis

RA is an autoimmune disease, which is characterized by synovial inflammation. In this inflammatory process, misdirected cells of the immune system immigrate in the joint and set inflammatory mediators free (cytokines). The cytokines can now dock on the receptors of the target cells and lead to equivalent reactions. They provide a generation of a swelling construct in the synovia, the pannus, which itself takes part in the destructive process and is resulting in the break down of cartilage tissue and bone material. Also osteoclast activation is increased, which is responsible for the reduction of bone material (Aryeh et al., 2006). This increase is also referred to elevated levels of RANKL, necessary activators for the osteoclasts.

Studies were also able to show that fibroblasts of the synovia are able to leave the affected tissue material and enter via blood circulation into healthy joint material (Lefévre et al., 2009). This process could explain the main symptom of the "migrating pain".

However, both OA and RA lead to a destruction of cartilage tissue and consequently to dysfunction and disability of the musculoskeletal system (Menche, 2007). Therefore, the symptoms for RA are very similar to OA, including tenderness, pain and swelling of the joint. Also stiffness is a main symptomatic evidence for RA that is occurring in longer periods than in OA, but also most likely in the mornings. The equivalent for the bone spurs in OA are in RA the rheumatoid nodules. Further symptoms are, because of the much higher rate of inflammation in RA symptoms like fatigue, fever and a loss of weight, but especially the characteristic migrating pain that can reach also unaffected joints of the body (Lefévre et al, 2009). Beside of the migrating symptoms, the batch-wise appearance of the disease, which leads to a variation of symptomatic intensity, is also characteristic.

1.3.1. Treatment for RA

Concerning RA, the therapeutic strategies are similar to OA, as there is neither a cure for it. The therapy is starting with lifestyle change, including physical therapy to prevent or slower the degenerative process of the cartilage tissue with the target to reduce symptomatic pain and increase life quality. And also in the process of RA, surgical intervention could be required if conservative therapy is not able to succeed.

The main difference in the therapeutic strategy between OA and RA is the medical intervention, as therapeutics for RA are not limited to analgesics.
The medical intervention includes drugs of different classes, which are besides of NSAIDs, disease modifying anti-rheumatic drugs (DMARDs), biologicals and glucocorticoids. NSAIDs have only symptomatic effects, as they are pain relievers. Contrary, DMARDs and biologicals are able to delay the progression of RA due to their immunosuppressive effects. Biologicals like Etanercept or Infliximap, are TNFα antagonists and are the most successful class of drugs in the medical intervention of RA (Aktories et al., 2009). However, the treatment of RA is very complex and an individual adaption of the therapy for each patient is necessary (Figure 1. 3.).

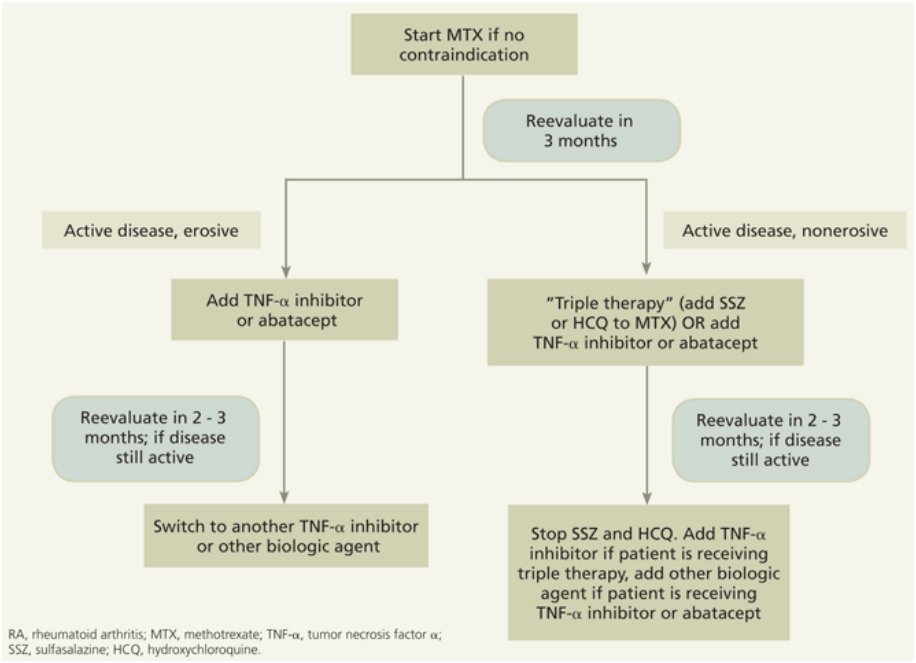

Figure 1. 3. Possible therapeutic approach of RA

(http://www.rheumatologynetwork.com/rheumatoid-arthritis/managing-ra-2011-update)

1.4. Herbal-medical therapy of OA and RA

Besides the conventional therapy of OA and RA, there is a range of alternative strategies that can be used to supplement therapeutic interventions for pain reduction. There are for instance the possibilities of laser therapy or homeopathy, for which no scientific study was able to demonstrate their effects, or acupuncture as symptom reducing intervention.

Herbal-medicine is also used to complement therapeutic strategies as nutritive supplement. Herbal substances like curcumin, also used as food dye (E100), is known to be a very potent anti-inflammatory, anti-oxidative, anti-carcinogenic and anti-amyloid agent. Curcumin is isolated from the plant Curcuma longa. It is very present in the traditional Indian kitchen as it gives the well known spice curry its characteristic flavor and color. Studies were able to show that curcumin interferes with the canonical nuclear factor-κB (NF-κB) pathways and consequently decreases gene expression levels of cyclooxygenase2 (COX2), MMP, TNFA and nitric oxide (Henrotin et al., 2013).

Dry extracts of Angelica sinensis are possibly providing protective benefit for articular cartilage. This plant finds its practice in the traditional Chinese medicine, in interventions against OA. Clinical studies indicated that components (sodium ferulate and polysaccharidic fraction) of Angelica sinensis prohibit destructive processes in OA and additionally promote cartilage tissue repair (Magdalou et al., 2015). That study showed that sodium ferulate successfully inhibited TNF-induced pathways and that polysaccharide fraction enhanced the production of proteoglycan, a major component of articular cartilage.

Another natural substance with high potential is resveratrol, a polyphenolic component found in plants like grapes, mulberries, raspberries or peanuts. Studies provided evidence that resveratrol was able to show a protective impact on chondrocytes by decreasing levels of inflammation, by antagonizing, at least partly, NFκB signaling pathways (Lju et al., 2014).

Diacerein is a chemical substance that is associated with the chemical class of antraquinone, it is contained in the plant Rheum rhabarbarum, the rhubarb. The substance was proven to be effective in the treatment of patients having knee OA (Pettelier et al., 2000), diacerin itself has pain reducing, anti-inflammatory and anti-arthritic features, that are resulting of its inhibiting effect on IL-1β, MMP, oxygenic radicals and proteolytic enzymes (Fidelix et al., 2014). As a prodrug it is rapidly metabolized in the human body to rhein, also a component of rhubarb. Rhein seems to promote the production of cells of the articular cartilage.

Peganum oil, tested in clinical trials in patients with knee OA showed that there was a significant decline of symptoms associated with OA. If the oil was rubbed on the knee in a period of four weeks, symptoms like swelling, pain and stiffness declined (Abolhassanzadeh et al., 2015). Peganum oil is yielded by the extraction of the plant Peganum harmala, which is found mainly in the deserts of West Asia. It offers a useful and uncomplicated opportunity of pain relieving therapy supplementation with surely high compliance rates.

The fruit of pomegranate, Punica granatum, is associated in many cultures with health supporting benefits. Also in modern medicinal therapies, products won of pomegranate fruit find their use, in treatments like against allergic symptoms, cancer (Kim et al., 2002) or cardiovascular circumstances. In the fruit two main substances can be found, anthocyanins as well as hydrolyzable tannins. Both components are of polyphenolic character and are responsible not only for the rich color of the fruit juice, but also for the antioxidative properties (Akhtar et al., 2012). Studies were able to show that punicalagin, a hydrolyzable tannin, was able to inhibit cyclooxygenase-2 enzyme activity and production rates of nitric oxide and prostaglandine E_2, which were induced in OA chondrocytes by IL-1β (Shukla et al., 2008). Overall, the literature indicates possible benefits of pomegranate fruit products in OA, but still needs evaluation (Khalifé et al., 2011).

1.5. Caesalpinia sappan

Another plant referred to the group of herbal-medical therapy products in OA and RA, is Caesalpinia sappan. The plant, depictured in Figure 1. 4., appears in Southeast Asia as a fast growing spreading tree or a bush with a height up to ten meters. It has been commonly used in its spreading area as a dying plant, but also found its use in local traditional medicine since the properties of the heartwood of Caesalpinia sappan (Figure 1. 5.), are reported as anti-inflammatory, analgesic and blood circulation improving.

Figure 1. 4. Caesalpinia sappan

(http://parisaramahiti.kar.nic.in/medicinal_plants_new/med%20plants/p26.html)

Figure 1. 5. Heartwood of Caesalpinia sappan

(http://www.yobo360.com/shop365/product-2359.aspx)

Studies showed that ethanolic extracts of Caesalpinia sappan were able to suppress in IL-1β-stimulated chondrocytes and lipopolysaccharide-stimulated THP-1 macrophages, expression levels of the inflammatory mediator IL-1β (Wu et al., 2011). In agreement, another study showed that brazilin, a main component of the plant, inhibits the activity of RA in type-II collagen stimulated mouse models (Jung et al., 2015). Consequently, further studies set a focus on the components of Caesalpinia sappan (unpublished data, Müller et al., 2015).

The main 5 substances of Caesalpinia sappan heartwood are episappanol, protosappanin C, brazilin, protosappanin B and sappanol (Figure 1. 6.).

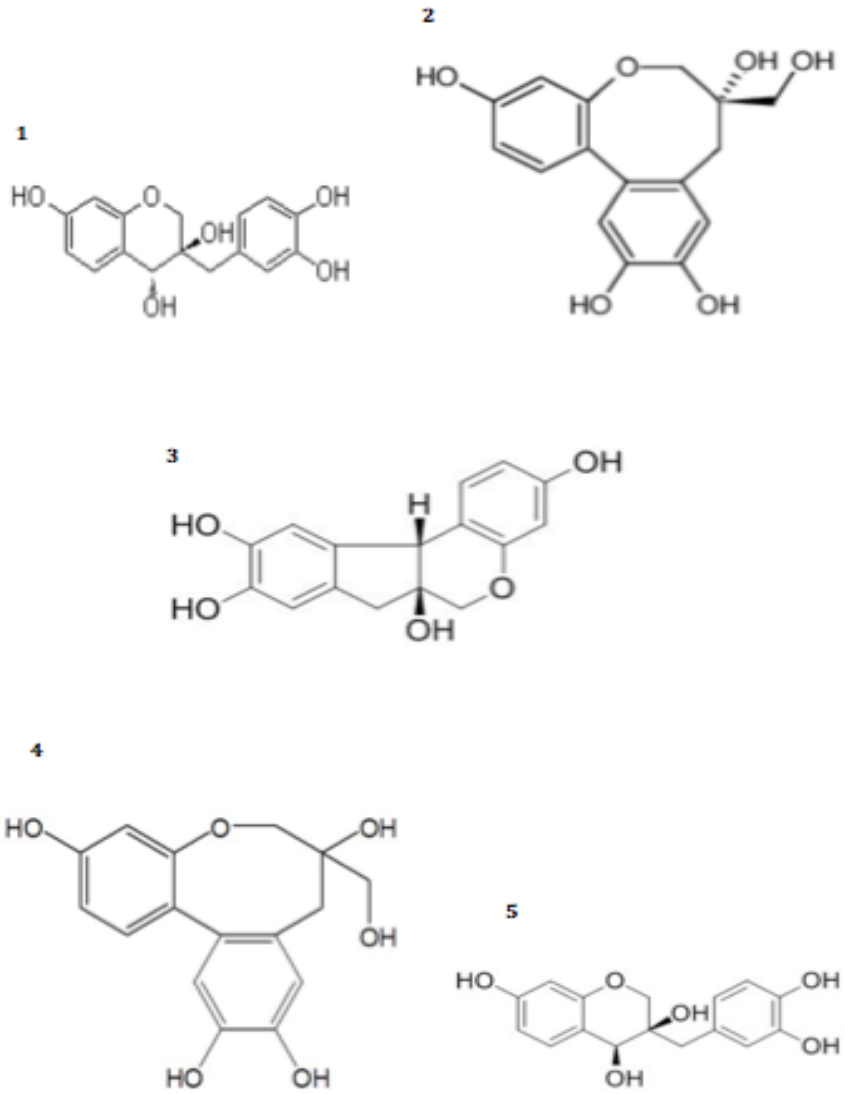

Figure 1. 6. The main 5 substances of Caesalpinia sappan heartwood

1:episappanol, 2: protosappanin C, 3: brazilin, 4: protosappanin B, 5: sappanol

Studies also provided evidence that brazilin activated heme oxygenase 1 protein synthesis in RAW264.7 macrophages (Hu et al., 2009). Consequently, it is reasonable to set the focus on the heme oxygenase 1 pathway and its interactions in further studies.

Heme oxygenase 1 (HMOX-1) is the enzyme that oxidases Heme to iron (Fe^{3+}), Biliverdin and carbon monoxide. Mammalians have three different isoforms of this enzyme (HMOX-1, -2, -3), which are found in the plasmosomes of the cells. Also cyanobacteria and red algae are possessing HMOX, it is essential for the porphyrin depletion, as well as for the signal transduction. Studies were able to show that HMOX-1 has a protective influence on chondrocytes (Clérigues et al., 2013). It was shown that the inflammatory

cytokine IL-1β led to an inhibition of HMOX-1 expression rates. This suppression resulted in catabolic effects, which were successfully reversed by HMOX-1 induction. Consequently, to the HMOX-1 induction, levels of MMP´s and TNF-α were normalized, as they were decreased.

1.6. Aims of the study

This thesis aimed to isolate the main components of Caesalpinia sappan, using high performance liquid chromatography and to investigate their effects on cultured human chondrocytes and synoviocytes of patients with OA and RA. First, we aimed to analyze the impacts on gene expression levels of IL1B, MMP13 and HMOX1, in presence and absence of pro-inflammatory IL-1β. Thereby, we sought to substantiate our hypothesis that brazilin might be responsible for the beneficial effects of Caesalpinia sappan extracts in OA. Moreover, particular focus was placed on the role of HMOX-1 in the biological activity of brazilin to assess the hypothesis that the effect of brazilin may correlate with induced HMOX-1 levels.

2. Material and Methods

2.1. **H**igh **P**erformance **L**iquid **C**hromatography (HPLC)

2.1.1. Source material & methods

Approximately 20 g minced pieces of the heartwood of Caesalpinia sappan were extracted with 200 ml absolute ethanol (Emplura) for 24 hours at room temperature under continuous stirring. The plant material of Caesalpinia sappan was provided by the Chiang Mai University of Thailand, heartwoods there are collected and identified according to vouchers specimen (No. 87-1631). After the maceration process the extract was filtrated and evaporated on the rotary evaporator (Heidolph VV2011). The dried residue was dissolved in 20% ethanol at a concentration of 100 mg/ml, centrifuged for 10 min with 13.000 revolutions per minute (rpm) and the supernatant was used for separation using preparative HPLC.

2.1.2. Preparative HPLC

Using preparative HPLC, the extract was separated in five fractions. Four runs were performed using the following HPLC system (Table 2. 1.).

System	Ultimate 3000
Stationary phase	Hypersil GOLD, 5 µm particle size 250 x 21,2 mm (Thermo Scientific)
Mobile phase	Eluent A: Water, 5% Acetonitrile Eluent B: Acetonitrile
Gradient	0.5% B (0 min), 17.5% B (30 min), 50% B (33 min), 100% B (34 min), 100% B (40 min), 0.5% B (41 min), 0.5% B (55 min)
Temperature	Room temperature
Detection wave length	254 nm, 280 nm
Flow rate	20 ml/min
Injection volume	1.000 µl, 1.500 µl

Table 2. 1. Preparative HPLC

2.1.3. The fractions

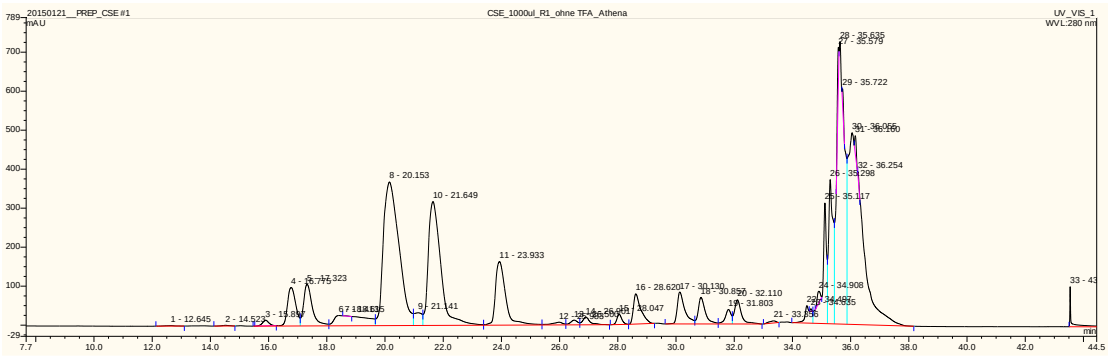

Figure 2. 1. The fractions

The fractions 1a (named fraction 1 in the following), 1b (was discarded after purity check), 2, 3, 4 and 5 were collected (Figure 2. 1.). All fractions were evaporated to remove the acetonitrile and filled in testing tubes. The testing tubes were cooled down to –20°C and lyophilized in its frozen state. After that, the lyophilized fractions were transferred into Eppendorf tubes and 0.2 mg were used for a purity check through an analytical HPLC.

2.1.4. Analytical HPLC

For the analysis of the plant extracts, the analytical reversed phase HPLC was used. Therefore, the samples were dissolved in dimethylsulfoixde (DMSO) at a final concentration of 1 mg/ml. The following HPLC system was used for the analysis (Table 2. 2.).

System	Ultimate 3000 RS (Thermo Scientific)
Stationary phase	Kinetex C-18.5 µm particle size, 100 Å, 150 x 4.6 mm (Phenomenex)
Mobile phase	Eluent A: Water (HPLC purity), 5% acetonitrile Eluent B: Acetonitrile
Gradient	0% B (0 min), 17.5% B (30 min), 50% B (33 min), 100% B (34 min), 100% B (35 min), 0% B (36 min), 0% B (38 min)
Temperature	40°C
Detection wavelength	254 nm, 280 nm
Flow rate	0.5 ml/min
Injection volume	20 µl

Table 2. 2. Analytical HPLC

Because of the unsatisfying purity of fraction 3 and 4, the preparative HPLC was repeated according to the afore mentioned method. This time, the preparative HPLC was conducted with an acidic eluent (addition of 0.1% trifluor acetic acid (TFA) to eluent A and B) for a better separation. In each case, the fraction with the highest purity (>90%) was used for biological assays.

2.2. Clinical specimens

Tissue of human articular cartilage was obtained from OA, RA and of one Ewing sarcoma patient, who all underwent surgical interventions within a total knee cartilage replacement therapy and in accordance with the terms of the ethics committee of the Medical University of Vienna (EKNr:081/2005). The excised joint material was obtained directly of the operating room, brought in small boxes containing a sterile physiological salt solution. For further cell culture procedure adequate parts of the joint were selected.

2.3. Cell culture

2.3.1. Isolation of primary chondrocytes and synoviocytes

The tissue material was obtained from the orthopedic surgery section of the Vienna General Hospital, received in sterile physiological salt solution.

In the following cell culture process, parts of cartilage tissue were dissected of the patella, femoral condyles and tibial plateau, in aim of isolating chondrocytes. For synoviocytes, synovial tissue was cut. The dissection was performed with sterile forceps (Ärztebedarf Scherer) and medical disposable scalpels (Heintel) on petri dishes (diameter of 10 cm, BD Falcon), in aim to mince cartilage in small pieces. The small pieces of cartilage were washed accurately with phosphate buffered saline (PBS). Subsequently, enzymatically digestion was performed using Collagenase B in a concentration of 2 mg/ml in full medium, which is Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS) and 1% gentamycin added. After continuous agitation over night at 37°C, chondrocytes were isolated from the suspension, resulting from cartilage digestion, using 40 µm nylon cell strainer (BD Falcon™). Afterwards, the cell suspension was centrifuged at 1.000 rpm for 10 min. The resulting cell pellet was resuspended in full medium and counted in a counting chamber (C-Chip Neubauer), to then seed them in culture flasks or well plates.

2.3.2. Cultivation of cells

Dependent on the number of synoviocytes and chondrocytes, which resulted from counting in a dedicated counting chamber, the cells were plated onto culture flasks or plates and cultivated at 37°C with 5% CO_2 and 95% relative humidity. The cells were morphologically observed and supplied with enough full medium.

When a confluent condition of 99% was reached the cells were split. Therefore, after a full medium exchange and washing step with PBS, required amount of trypsin was added for about 4 min, depending on the volume of culture flaks (as shown in Table 2. 3.).

Culture flask/ Well plate	Surface	Required amount of full medium	Required amount of trypsin / Used for celltype
T25	25 cm^2	5 ml	1 ml
T75	75 cm^2	15 ml	2 ml
T150	150 cm^2	30 ml	4 ml
6-well plate	9.5 cm^2	2 ml per well	Chondrocytes
12-well plate	3.8 cm^2	1 ml per well	Synoviocytes
96-well plate	1 cm^2	100- 200 µl	Chondrocytes and synoviocytes

Table 2. 3. Qualities of used culture flasks and plates

By passaging the cells to different flasks and plates, a cellular dedifferentiation process is caused. This is observable by a modification of phenotypic and genotypic character of chondrocytes and synoviocytes. Consequently, chondrocytes are used at passage level 0 and synoviocytes at a passage level of 4 until 8, at this levels the cells provide still characteristics of their cell kind, so outcomes of cell treatments can be correlated also to cell type.

The treatment of chondrocytes and synoviocytes was performed in 6-well or 12-well plates (Table 2. 3.). Therefore, a washing step with PBS was performed prior to starvation of the cells. Synoviocytes were starved for 6 hours and chondrocytes overnight. All cells were treated for 24 h with fractions of CSE or pre-treated with fractions of CSE and co-incubated for 24 h with IL-1β. Additionally, cells without treatment and with only cytokine treatment were cultivated under same conditions as controls.

2.3.3. Freezing and thawing of synoviocytes

To conserve synoviocytes with the aim of integrating them at a later time point into the working process, they can be frozen. In contrast, chondrocytes were used only freshly isolated without passaging, as this kind of cells would start to dedifferentiate if passaged. In order to freeze synoviocytes they were detached with trypsin as mentioned in → 1.2.2. Cultivation of cells, prior to centrifugation and resuspending the pellet full medium was supplemented in required amount. The resulting cell suspension was transferred in a cryotube, supplemented with 100 µl dimethyl sulfoxide (DMSO; SIGMA) as cryoprotective agent. An optimal cooling rate of − 1°C per minute, which is achievable with Mr.Frosty Freezing container, was applied. With this cooling system, the formation of ice crystals can be prevented before transferring the synoviocytes in the nitrogen tank for cryopreservation, which guarantees maximal sample protection.

To thaw the cell suspension at minimum time, the cryotube was taken out from the nitrogen tank and transferred in a warm water bath. The thawing cell suspension was added to 9 ml full medium and centrifuged for 8 minutes at 1.000 rpm at 20°C to remove the DMSO. The final pellet was resuspended in full medium and the synoviocytes were seeded in the requested flasks or plates.

2.3.4. Non-radioactive cell proliferation and cytotoxicity assay

The EASY FOR YOU (EZ4U) assay (Biomedica) is based on a chemical reaction, the reduction of yellow tetrazolium into its red colored metabolite, a formazan derivate. This reaction is performed by living cells. If these cells are dead their mitochondria get inactivated rapidly and the main requirement for the terazolium metabolize is lost. Due to this fact, it was possible to achieve knowledge of compatible concentrations of the five fractions of CSE on synoviocytes and chondrocytes.

Cell suspensions of synoviocytes and chondrocytes were seeded in 96-well plates (Table 2. 3.) and cultivated until a confluence of 99% was achieved. Afterwards, the cells were not starved overnight but treatment was performed in starvation medium. Treatment was performed overnight with an established dilution series of every fraction with concentrations of 100, 50, 25, 10, 5, 2 µg/ml, added in duplicates. Subsequently, culture medium was exchanged with 200 µl fresh starvation medium and supplementary 20 µl dye solution were added, following the manufactures protocol. Cells were incubated for 3 h and absorbance was measured at 450 nm with 620 nm as reference, using microtiterplate readers VICTOR^3 1420 Multilabel Counter or FLUOstar OPTIMA BMG LABTECH with the EASY FOR YOU program (EZ4U, the 4th generation non-radioactive cell proliferation and cytotoxicity assay). It measures the absorbance of the red colored formazan in the cell culture supernatant.

2.3.5. RNA isolation

Working procedure was performed according to NucleoSpin RNA protocols (Macherey-Nagel; RNA purification from cultured cells and tissue).

After cell treatment, the lysis was started. For that, after cells were washed with PBS, 350 µl Buffer RA1 supplemented with 3.5 µl β-mercaptoethanol (Sigma-Aldrich) were added to chondrocytes and synoviocytes in every well and after pipetted up and down until the viscosity was reduced.

If the working process had to be interrupted, the cell lysate was frozen (–80°C).

Then, the lysate was filtrated through NucleoSpin Filter (violet ring) with 11.000 rpm for 1 min. To adjust the RNA binding conditions, the NucleoSpin Filter was discarded and 350 µl 70% ethanol was added. After pipetting the lysate up and down, it was loaded for the RNA binding on NucleoSpin RNA Column (light blue ring), which was placed in a Collection Tube and centrifuged for 30 s at 11.000 rpm. Before the DNA was digested, the silica membrane had to be desalted. For that, 350 µl Membrane Desalting Buffer (MDB) was applied and centrifuged at 11.000 rpm for 1 min. Then, the prepared DNase reaction mixture, containing 10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase, was loaded centrally on the membrane. After incubation time of 15 min, the silica membrane was washed and dried. This process was considering 3 steps with 200 µl Buffer RAW2, 600 µl RA3 and 250 µl RA3 and intermediate centrifuging. The final RNA elution was performed in 60 µl nuclease-free water (Ambion THE RNA COMPANY) and centrifuged at 11.000 rpm for 1 min.

2.3.6. RNA quantification

Using NanoDrop 2000c UV-Vis spectrophotometer and the corresponding software, (Thermo Scientific) the quantity and purity (260/280 ratio) of isolated RNA material was measured. For that, 2 µl of the vortexed samples were used.

The Beer-Lambert Law is representing the foundation of spectrophotometry and allows the calculation of nucleic acid concentrations. In this process, the lowest concentration is set in relation to the remaining results. The following results provide the needed information how much water has to be added to achieve a standard concentration within of one experiment.

2.3.7. cDNA synthesis

For further quantitative polymerase chain reaction (PCR), total RNA was reverse transcribed into single-stranded cDNA. Therefore, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. Obtained components (volumes per sample) are shown in Table 2. 4..

Component	Volume (µl)
10xRT Buffer	2.0 µl
25xdNTP Mix (100mM)	0.8 µl
10xRT Random Primers	2.0 µl
MultiScribe Reverse Transcriptase	1.0 µl
Nuclease-free H_2O	4.2 µl
Total per reaction	10 µl

Table 2. 4. Components of High Capacity cDNA Reverse Transcription Kit

Every sample was supplemented with 10 µl of reverse transcription master mix (mixture of High Capacity cDNA Reverse Transcription Kit components). Afterwards, after the 20 µl reaction volume was loaded in tubes on the thermal cycler (Eppendorf Mastercycler Personal).

The started reversed transcription program included 4 steps, as seen in Table 2. 5..

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

Table 2. 5. Eppendorf Mastercycler program

2.3.8. Quantitative real time RT-PCR

Quantitative real time polymerase chain reaction (RT-qPCR) was performed for IL1B, MMP13, HMOX1. Succinate dehydrogenase complex (SDHA) was used as housekeeping gene, which was expressed independently from external conditions. 1 reaction required 24 µl of master mix, components shown in Table 2. 6..

Component	Volume (µl)
Forward primer	0.5 µl
Reverse primer	0.5 µl
SensiMix SYBR MASTER mix (Bioline)	12.5 µl
H_2O	10.5 µl
Total per reaction	24 µl

Table 2. 6. Components of the RT-qPCR master mix

The master mixtures were pipetted into a Fast Optical 96-Well Reaction Plate (Applied Biosystem) and supplemented with 1 µl of, with H_2O 1:5 diluted, cDNA sample. All samples were pipetted in duplicates. After applying Optical 8-Cap Strips (Applied Biosystems) onto the plate, it was put in the StepOne Plus qPCR instrument (Life Technologies, USA).

SYBR Green is a fluorescent dye, which is binding to double-stranded DNA, PCR produced molecules. Within each cycle increased fluorescents, resulting of the PCR product, is measured (Table 2. 7.).

Generated melting curves confirmed a single gene-specific peak. And after the threshold setting the regulated target genes are calculated as relative quantities to the control.

Holding stage	Step1	95°C	10 min	/
Cycling stage (40 cycles)	Step 1	95°C	15 s	/
	Step 2	55°C	1 min	Measurement
	Step 3	72°C	1 min	Measurement
Melt curve stage	Step 1	95°C	15 s	/
	Step 2	55°C	1 min	/
	Step 3	95°C	15 s	Measurement

Table 2. 7. RT-qPCR program

2.3.9. Western blot

For separation of proteins sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. In the blotting process, separated protein bands were transferred on a nitrocellulose membrane (Schleicher & Schuell), on which detection by target specific antibodies took place. Therefore, a particular transfer cassette order was followed, as described in the following: black side- black sponge- filter paper- white sponge- white side. The proteins were transferred for 2 h (at 250 mA) in a Mini Trans-Blot Cell (Biorad), which contained transfer-Buffer (compounded of 700ml H_2O, 200 ml methanol, 100 ml 10x Tris-Glycine-Buffer pH 8.4). Afterwards, the membrane with the transferred proteins underwent a blocking process with 5% (w/v) milk powder (Roth) which was dissolved in 1x PBS pH 7.4 overnight at a temperature of 4°C under continuous shaking.

Then, the membrane was washed with PBS-T (compounded 1x PBS, 0.1% (v/v) Tween20 (Biorad)) for 10 min in 3 washing steps. The followed detection of proteins was performed by incubating the membrane at room temperature for 2 h on a roller mixer with primary Anti-Heme Oxygenase 1 antibody (rabbit polyclonal to Heme Oxygenase 1) and α-tubulin mouse monoclonal antibody (reference).

Afterwards membrane was washed with the same washing procedure with PBS-T. Secondary antibodies, Dylight 800-conjugated anti-rabbit IgG made in goat and IRDye 680LT anti-mouse IgG made in goat, diluted 1:15,000 in PBS-T, added 15 µl 20% SDS, were applied within a 1 h incubation at room temperature, on a roller mixer, protected from light. Again, washing steps were repeated with PBS-T. Finnaly, protein bands were visualized using the Odyssey Imager (Licor).

2.4. Statistics

Using paired Student´s t-test, with p-values < 0.05 considered as statistically significant, statistical analysis were performed.

3. Results

3.1. Separation of Caesalpinia sappan extract in its fractions

The ethanolic extract of Caesalpinia sappan (CSE) was separated in its main five fractions, whereby every fraction contained one pure substance. For this separation process preparative HPLC was performed → 1.1.2. Preparative HPLC. All five substances were separated due to their specific retention time, as shown in Figure 3.1..

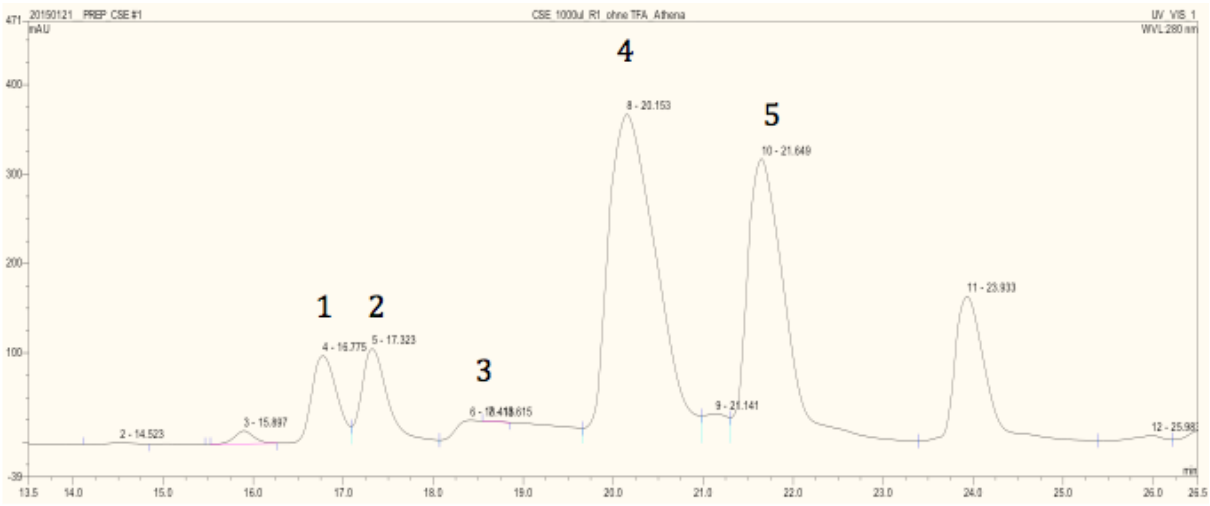

Figure 3. 1. Separated fractions of CSE

Extracted plant material was separated using preparative HPLC. Fraction one contained episappanol and showed a retention time of 16.8 minutes. Fraction two contained protosappanin C with a retention time of 17.2 minutes. Fraction three was collected due to its retention time after 20.2 minutes and included brazilin. Protosappanin B was contained in fraction four with a retention time of 21.6 minutes. Fraction five, sappanol, was collected after 23.9 minutes.

After preparative HPLC, the separated fractions were proceeded in the following resulting yields, fraction one: 2.05 mg, fraction two: 1.93 mg, fraction three: 11.93 mg, fraction four: 5.95 mg and fraction five: 3.92 mg (Figure 3.2.).

Figure 3. 2. Resulting yields from fraction one to five in their lyophilized state.

3.2. Purity check of CSE fractions

Purity check of all five fractions was performed using analytical HPLC. Every fraction was obtained with a purity of over 90%, which is required for biological assays.

3.3. Non-radioactive cell proliferation and cytotoxicity assay

To investigate how fractions of CSE affect the proliferation process in cells, the EZ4U assay was performed → 2.3.4. Non-radioactive cell proliferation and cytotoxicity assay. This assay is based on the finding that living cells have the ability to reduce yellow colored salts of tretrazolium into intensively red colored formazan deviates. For this process, cells need functional mitochondria. Because mitochondria are inactivated shortly after cell death, this method allows a differentiation between living and dead cells.

For the proliferation assay, cells of three patients were analyzed. The analyzed cells were of three different types, namely OA chondrocytes, OA synoviocytes and RA synoviocytes and were treated in duplicates with 2 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml of each fraction. For comparison, untreated cells were used as control (100% mitochondrial activity).

Based on this results, the concentrations of fractions were adjusted for the following treatments to ensure cell viability in treatment processes.

The following three figures illustrate mitochondrial activity under a treatment with CSE fractions at different concentrations.

3.3.1. Non-radioactive proliferation and cytotoxicity assay of OA chondrocytes

It was observable that in direct comparison to the control, which showed mitochondrial activity of 100%, each fraction drastically decreased the metabolic activity starting at a concentration over 10 µg/ml, brazilin even earlier with concentrations over 5 µg/ml. In comparison to a 10 µg/ml concentration (in case of brazilin compared to 5 µg/ml) the metabolic capacity of OA chondrocytes was reduced to rates of 30%. Compared to the untreated control, a statistical significance was reached for the treatment with 50 µg/ml brazilin and with 25 µg/ml, 50 µg/ml, 100 µg/ml sappanol.

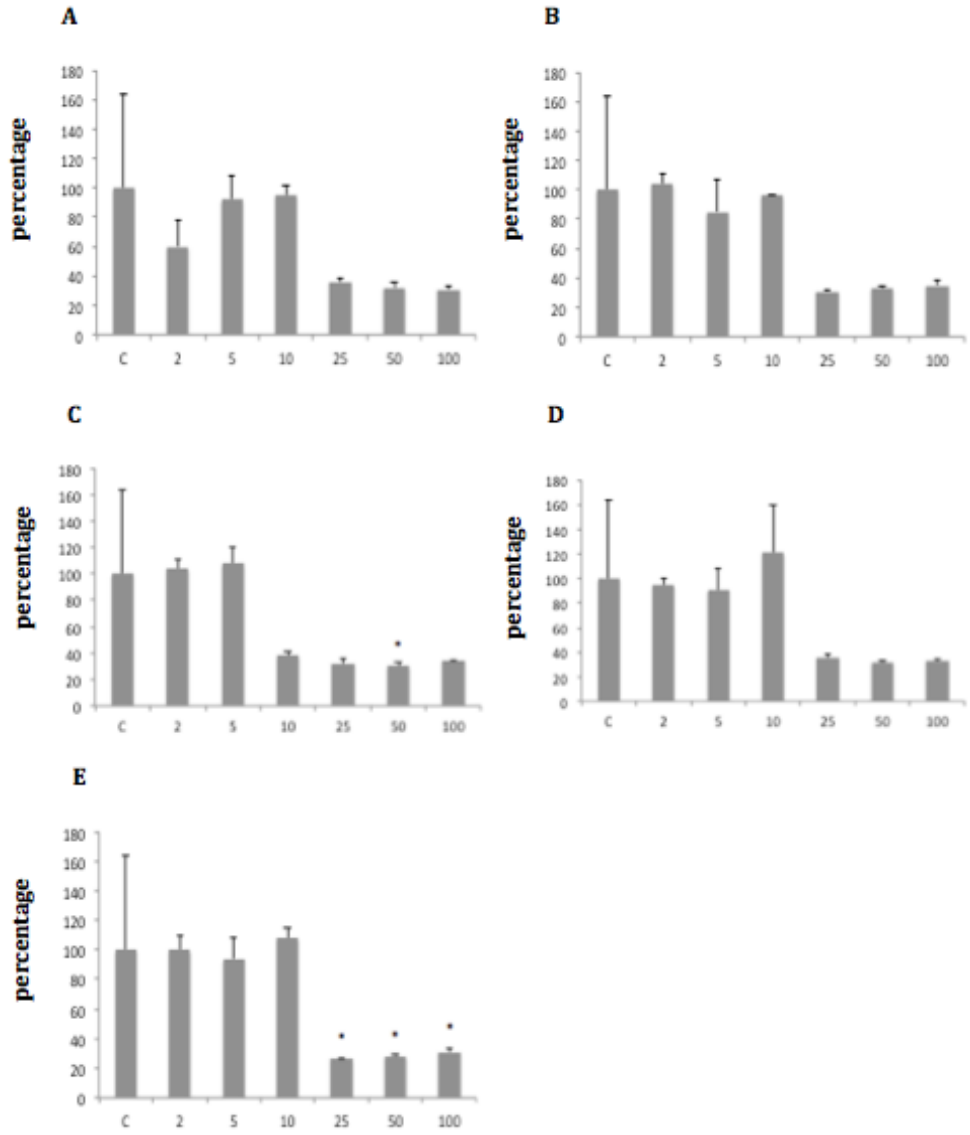

Figure 3. 3. Non-radioactive cell proliferation and cytotoxicity assay of OA chondrocytes Cells were treated for twenty-four hours with **A** episappanol, **B** protosappanin C, **C** brazilin, **D** protosappanin B and **E** sappanol in different concentrations (2/ 5/ 10/ 25 /50 / 100 µg/ml). As reference, untreated OA chondrocytes (C) were cultivated under same conditions. Asterisks mark a statistical difference of mitochondrial activity, compared to the control. P-values < 0.05 were considered significant (determined by t-test).

3.3.2. Non-radioactive proliferation and cytotoxicity assay of OA synoviocytes

It was observable that in direct comparison to the control, which showed mitochondrial activity of 100%, each fraction drastically decreased the metabolic activity at a concentration of 100 µg/ml, brazilin even earlier with concentrations over 5 µg/ml. Compared to the untreated control, a statistical significance was reached for the treatment with 100 µg/ml episappanol, 100 µg/ml protosappanin C, 25/ 50/ 100 µg/ml brazilin, 10/ 25/ 100 µg/ml protosappanin B and with 5/ 10/ 25/ 50/ 100 µg/ml sappanol.

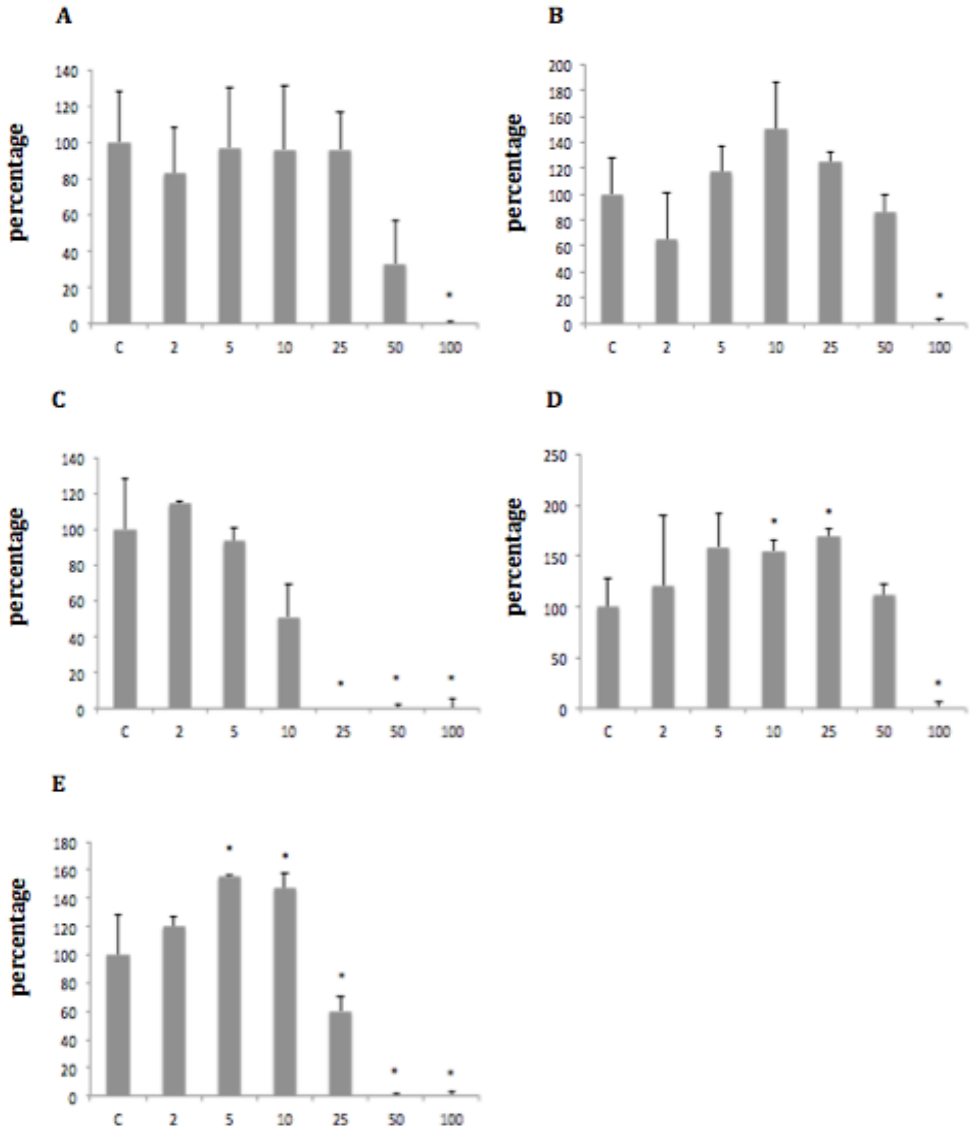

Figure 3. 4. Non-radioactive cell proliferation and cytotoxicity assay of OA synoviocytes Cells were treated for twenty-four hours with **A** episappanol, **B** protosappanin C, **C** brazilin, **D** protosappanin B and **E** sappanol in different concentrations (2/ 5/ 10/ 25/ 50/ 100 µg/ml). As reference untreated OA synoviocytes (C) were cultivated under same conditions. Asterisks mark a statistical difference of mitochondrial activity, compared to the control. P-values < 0.05 were considered significant (determined by t-test).

3.3.3. Non-radioactive proliferation and cytotoxicity assay of RA synoviocytes

It was observable that in direct comparison to the control, which showed mitochondrial activity of 100%, each fraction drastically decreased the metabolic activity starting at concentrations over 10 µg/ml, protosappanin C later with over 25 µg/ml. In comparison to a concentration of 10 µg/ml, brazilin significantly inhibited the activity down to 0 % with concentrations over 10 µg/ml. Compared to the untreated control, a statistical significance was reached for the treatment with 50/100 µg/ml episappanol, 50/ 100 µg/ml protosappanin C, 5/ 25/ 50/ 100 µg/ml brazilin, 2/ 25/ 50 µg/ml protosappanin B and with 25/ 50/ 100 µg/ml sappanol.

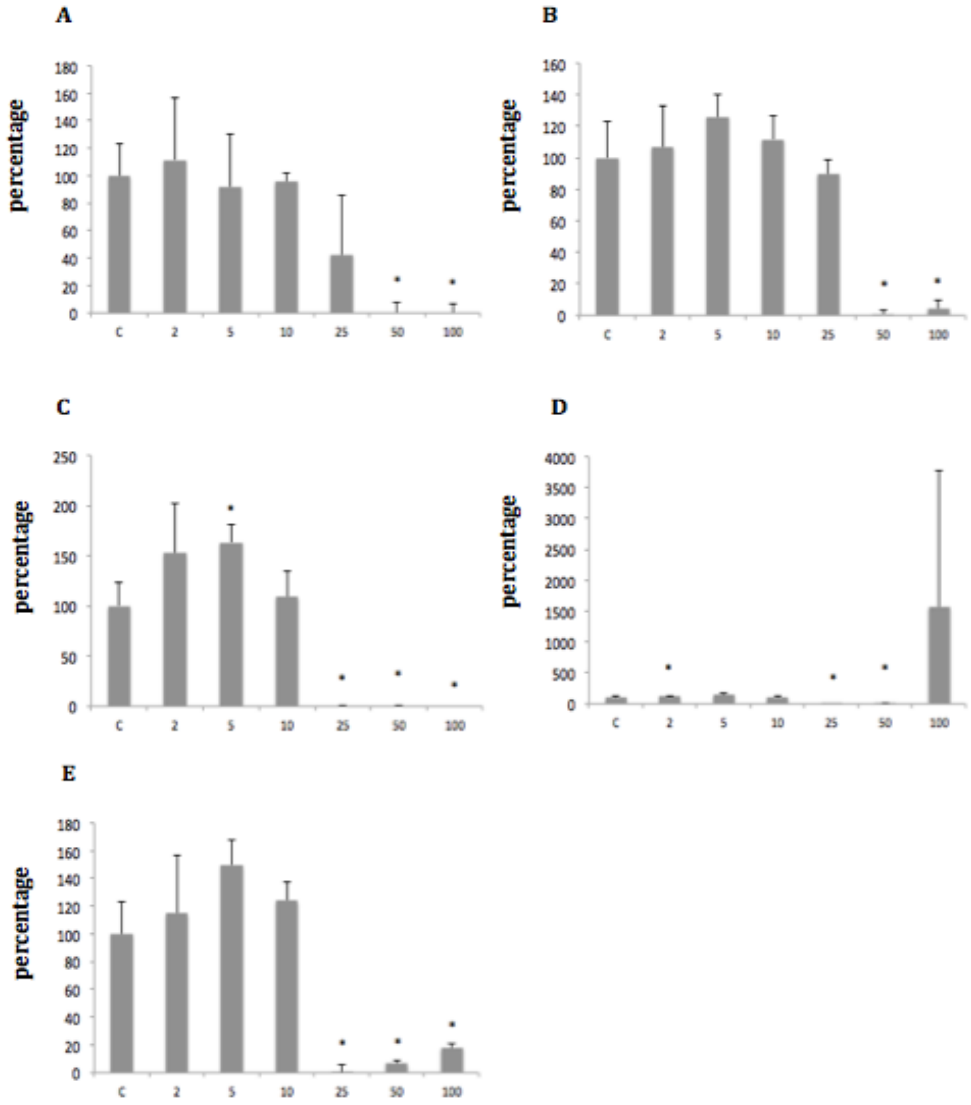

Figure 3. 5. Non-radioactive cell proliferation and cytotoxicity assay of RA synoviocytes Cells were treated for twenty-four hours with **A** episappanol, **B** protosappanin C, **C** brazilin, **D** protosappanin B and **E** sappanol in different concentrations (2/ 5/ 10/ 25/ 50/ 100 µg/ml). As reference, untreated RA synoviocytes (C) were cultivated under same conditions. Asterisks mark a statistical difference of mitochondrial activity, compared to the control. P-values < 0.05 were considered significant (determined by t-test).

3.4. mRNA level quantification in CSE fraction treated cells

To analyze the effects of the isolated compounds on IL1B, MMP13 and HMOX1 expression levels, qPCR was performed on CSE fraction treated human chondrocytes as well as on human synoviocytes.

Considering the outcomes of performed non-radioactive cell proliferation and cytotoxicity assay (→ 3.3. Non-radioactive cell proliferation and cytotoxicity assay), the treatment procedure for chondrocytes included an overnight starvation, followed by pre-treatment with 10 µg/ml of each fraction for one hour and subsequent 24-hour treatment with 10 ng/ml IL-1β. For synoviocytes, the procedure included a six-hour starvation and a 5 µg/ml pre-treatment with CSE fraction for one hour and an overnight co-incubation with 10 ng/ml of IL-1β.

Regarding RNA isolation, quantification and cDNA synthesis, the work processes for chondrocytes and synoviocytes were identical. cDNA was used for the investigation of gene expression levels in qPCR assays (→ 2.3.5. RNA isolation, 2.3.6. RNA quantification, 2.3.7. cDNA synthesis).

3.4.1. mRNA levels of OA chondrocytes

The following six figures show the gene expression level of IL1B, MMP13 and HMOX1 in human OA chondrocytes treated with fractions of CSE, either alone or following additional stimulation with IL-1β.

Figure 3. 6. illustrates the influence of each fraction on the mRNA level of HMOX1 in direct comparison to the control. In the performed experiment, brazilin (F3) significantly up-regulated HMOX1 (p=0.04). Compared to the control, 10 µg/ml brazilin were able to induce a 26.12±6.91-fold increase. This high increase was visible in all three patients. In contrast, the other compounds showed no effect.
Because HMOX1 is known to be an anti-inflammatory and anti-oxidative enzyme and was up-regulated by brazilin, we further analyzed the gene expression levels under inflammatory conditions.

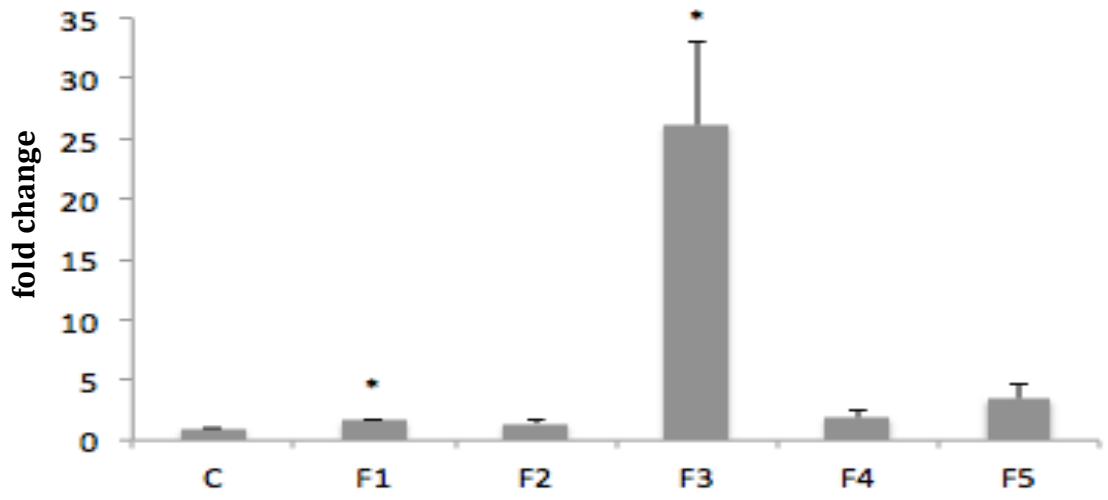

Figure 3. 6. HMOX1 mRNA levels of OA chondrocytes after treatment with CSE fractions

A twenty-four hour treatment was performed with chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA chondrocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 7. shows the mRNA levels of HMOX1 under the influence of the five fractions of CSE under inflammatory culture conditions. As such, qPCR was performed to analyze if HMOX1 mRNA levels also increased following addition of 10 ng/ml IL-1β. In comparison to the control, brazilin was again able to strongly up-regulate HMOX1 within cells of each patient, resulting in an overall 52.97±36.11-fold increase. However, it did not reach statistical significance across all patients due to inter-individual variability. IL-1β with a 2.15±1.8-fold increase showed no significant influence on HMOX1 levels. Taken together, brazilin caused an up-regulation of HMOX1 even under inflammatory cytokine treatment. Due to this finding, further qPCRs were performed to determine if this effect was also shown for gene expression levels of IL1B and MMP13.

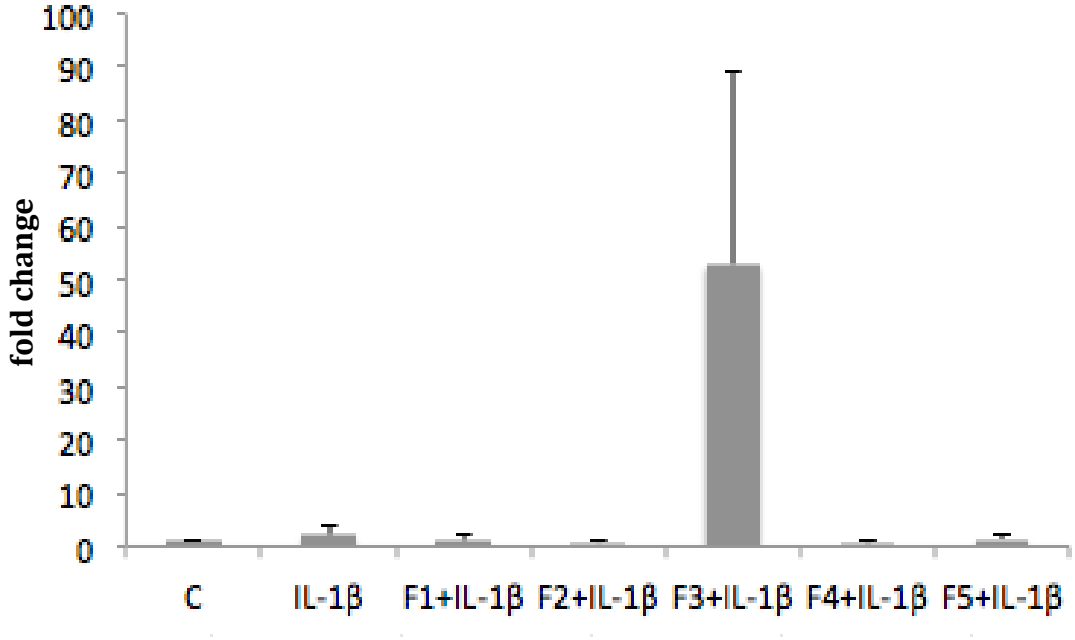

Figure 3. 7. HMOX1 mRNA levels of OA chondrocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE, followed by a twenty-four-hour co-incubation with 10 ng/ml IL-1β. As reference, untreated OA chondrocytes (C) and chondrocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 8. indicates the gene expression levels of the OA marker IL1B under the influence of CSE fractions. None of the five fractions showed a significant difference in direct comparison to the untreated control cells. The range extended from 1.12±0.16 (F4) to 1.53±0.58 (F3). Due to inter-individual differences and the small extent of gene regulation, there was no statistical significance across all three patients.

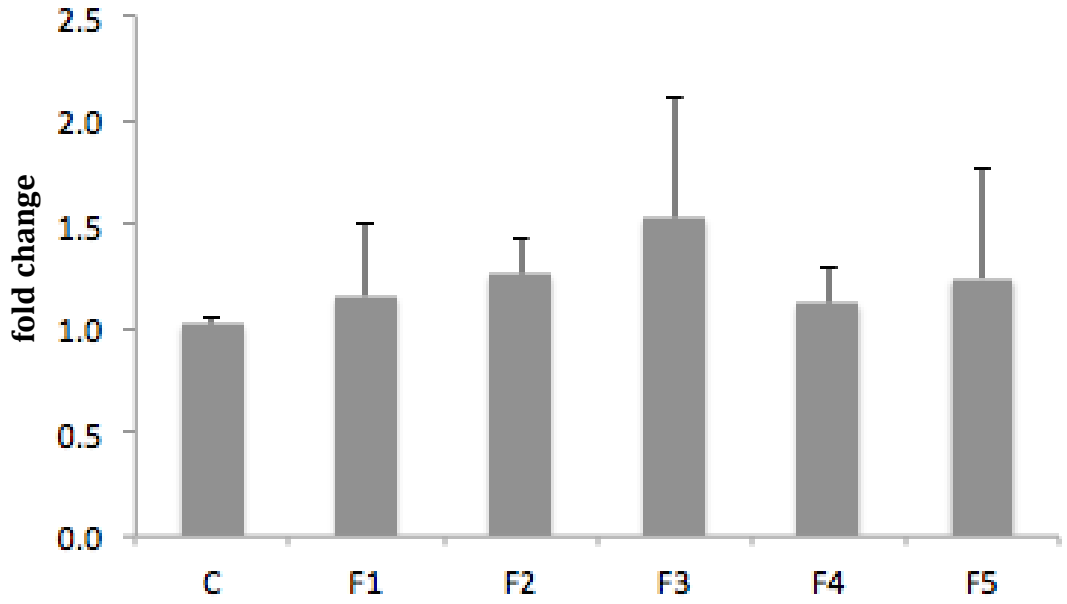

Figure 3. 8. IL1B mRNA levels of OA chondrocytes after treatment with CSE fractions

A twenty-four hour treatment was performed on chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA chondrocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 9. displays the regulative capacity of the CSE fractions in presence of IL-1β regarding mRNA levels of the pro-inflammatory cytokine IL1B. The cytokine control (IL-1β) showed that a treatment of cells with 10 ng/ml IL-1β led to a 134.9±59.97-fold up-regulation of the marker, showing that inflammatory conditions were induced by IL-1β stimulation. However, in comparison to the cytokine control, fraction three (brazilin) was able to push cytokine levels down, with an almost statistical significance of p=0.06 (compared to IL-1β), to a basal levels (to 1.26±0.47) compared to untreated OA chondrocytes.

After showing the anti-inflammatory effect of brazilin, qPCR experiments for MMP13 were performed to investigate if this matrix-degenerative enzyme was also down-regulated by this agent.

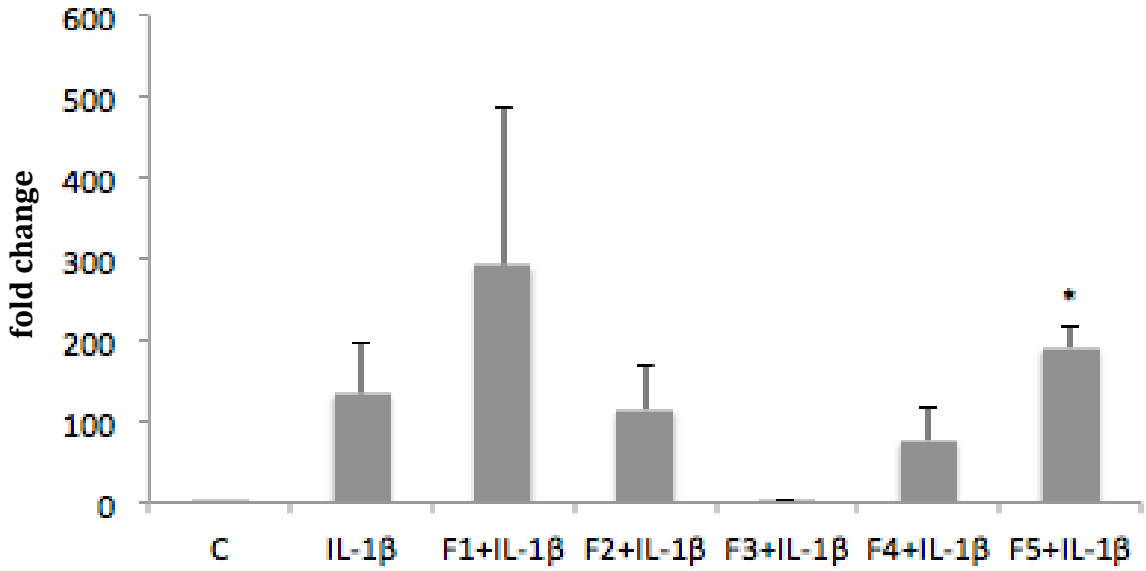

Figure 3. 9. IL1B mRNA levels of OA chondrocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE, followed by a twenty-four-hour co-incubation with 10 ng/ml IL-1β. As reference, untreated OA chondrocytes (C) and chondrocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistical significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 10. presents the MMP13 mRNA levels following treatment with 10 µg/ml of the isolated compounds. The gene expression influenced by the compounds was comparable to the control with a range between 0.72±0.38 (F3) and 1.19±0.54 (F5). Throughout all five fractions, there was no statistical significance detected, due to inter-individual variability within all patients.

In the following, MMP13 gene expression levels were analyzed in cells under inflammatory conditions.

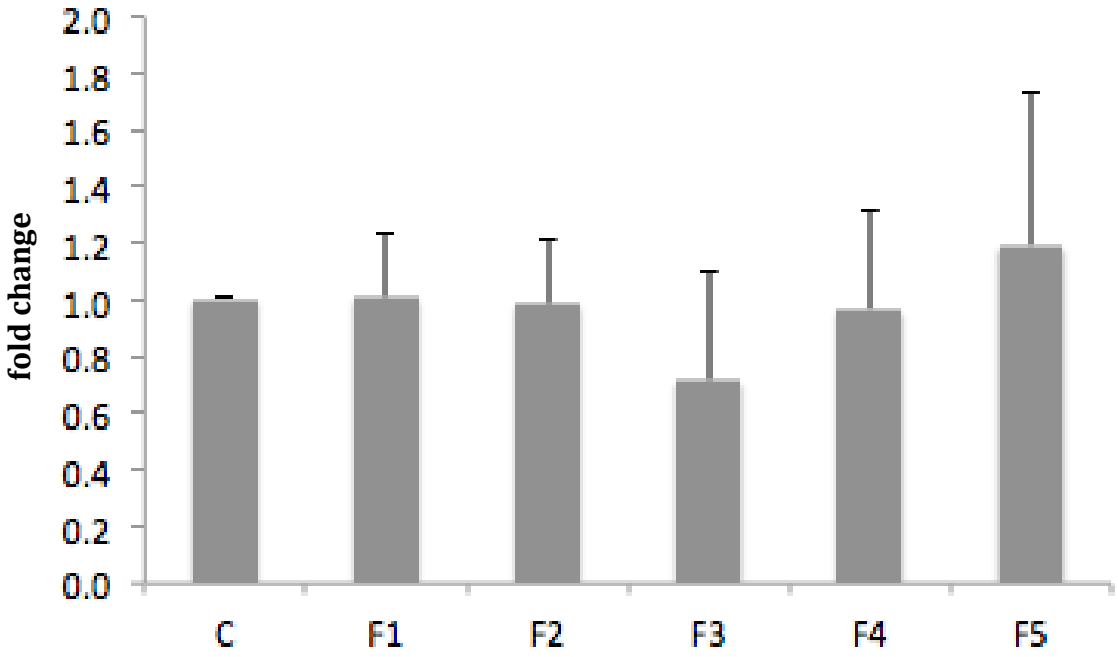

Figure 3. 10. MMP13 mRNA levels of OA chondrocytes after treatment with CSE fractions

A twenty-four hour treatment was performed on chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA chondrocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 11. shows the MMP13 expression in presence of IL-1β and following a pre-treatment with 10 µg/ml of CSE fractions. IL-1β, compared to the control, led to a 13.88±6.65-fold increase of the MMP13 mRNA level (p=0.08). Within a range of 1.23±0.96 (F3) to 17.06±10.95 (F1), brazilin (F3) was clearly able to decrease the MMP13 mRNA level to a basal level in direct comparison to IL-1β (p=0.08).

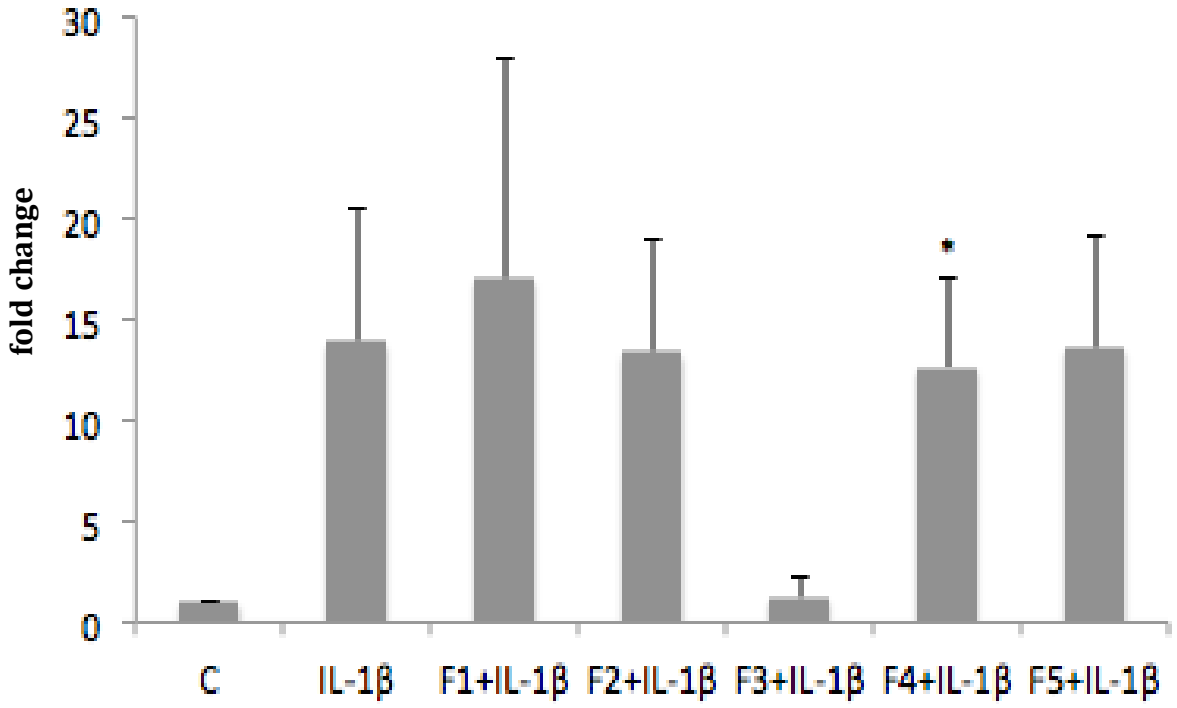

Figure 3. 11. MMP13 mRNA levels of OA chondrocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on chondrocytes of OA patients (n=3) with 10 µg/ml of five different fractions (F1-5) of CSE, followed by a twenty-four-hour co-incubation with 10 ng/ml IL-1β. As reference, untreated OA chondrocytes (C) and chondrocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistical significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

3.4.2. mRNA levels of OA synoviocytes

Having shown that CSE fractions influenced expression levels of the marker genes HMOX1, IL1B and MMP13 in OA chondrocytes, further qPCR experiments were performed with OA synoviocytes treated with the same compounds.

With the following twelve figures, we aimed to evaluate if the isolated compounds induce reactions in OA synoviocytes that are comparable with those observed in OA chondrocytes.

Figure 3. 12. illustrates how fraction one to five of CSE influenced the HMOX1 expression levels in OA synoviocytes. The cells were stimulated for twenty-four hours with 5 µg/ml of each fraction. The resulting gene expression range showed a minimal change of 1.13±0.25-fold (F1) and a maximal change of 5.07±0.31-fold (F3). The qPCR experiment showed that brazilin (F3) was able to increase mRNA levels of HMOX1 5.07±0.31-fold with a statistical significance in direct comparison to the control. In comparison to the control, also fraction five significantly up-regulated HMOX1 levels 2.29±0.35-fold. Likewise, also protosappanin C (F2) showed an increase of 2.24±0.55-fold with a significance level of p=0.06. In contrast, fraction one and four showed only slight and non-significant modifications of the gene expression level, compared to the untreated control.

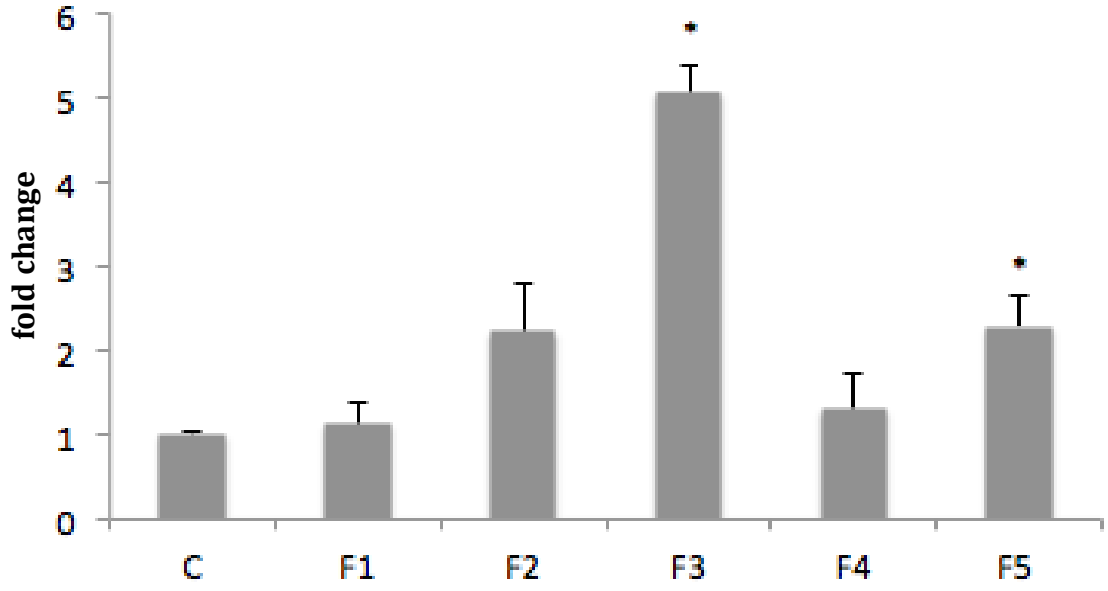

Figure 3. 12. HMOX1 mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of OA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1 qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistical significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

For yet undetermined reasons, OA synoviocytes of one patient showed the same HMOX1 expression pattern as in Figure 3. 12., but in much higher dimensions (→ Figure 3. 13.). Therefore, this qPCR experiment was analyzed separately. The range extends from a modification of 4.05±0.39-fold (F1) to 61.56±1.94-fold (F3). Brazilin (F3) was able to significantly increase the expression level 61.56±1.94-fold, as compared to the control. In contrast, not significant in comparison to the untreated control was the change of mRNA levels of the other four fractions, fraction one with 4.05±0.39-fold, fraction two with 7.02±0.17-fold, fraction four with 7.89±0.91-fold and fraction five with 10.1±0.36-fold modifications.

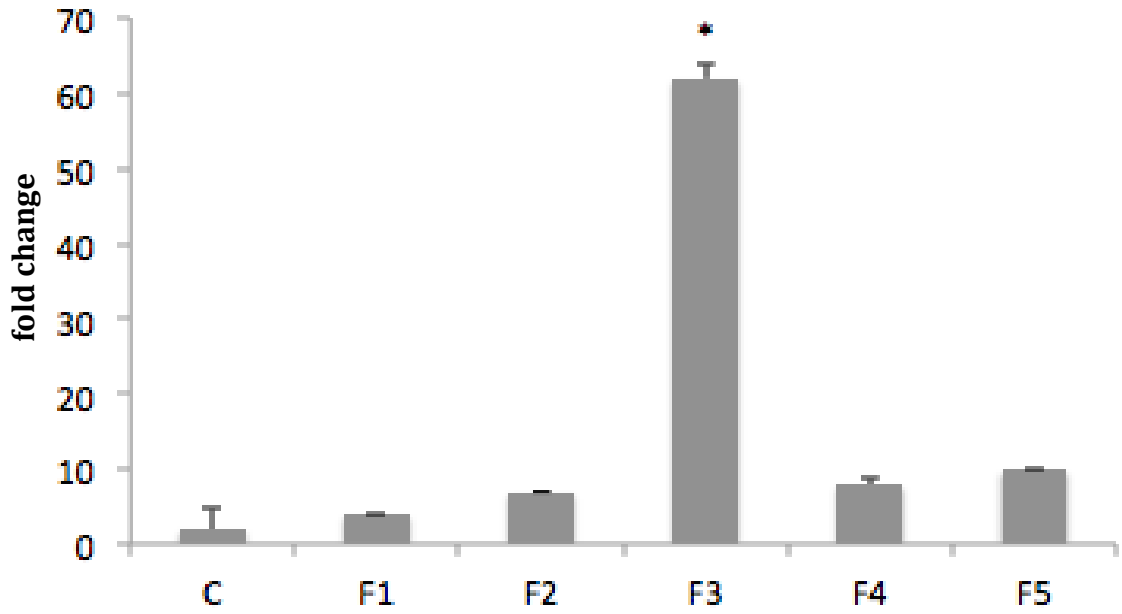

Figure 3. 13. HMOX1 mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistical significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

To analyze if HMOX1 levels are influenced in the same way under inflammatory conditions, qPCR experiments were performed in presence of the cytokine IL-1β.

Figure 3. 14. shows how the expression levels of HMOX1 alter in IL-1β-stimulated OA synoviocytes. In presence of the cytokine, HMOX1 levels stayed unaffected. There was no statistical significance for IL-1β and throughout all five fractions because of the inter-individual variability of all three patients. The range starts at a minimum of 1.01±0.71-fold change for F4 and ends at a maximum of a 2.19±2.1-fold change induced by fraction five in presence of the cytokine.

Also in the qPCR series with IL-1β presence, one experiments had to be analyzed separately (→ Figure 3.15.).

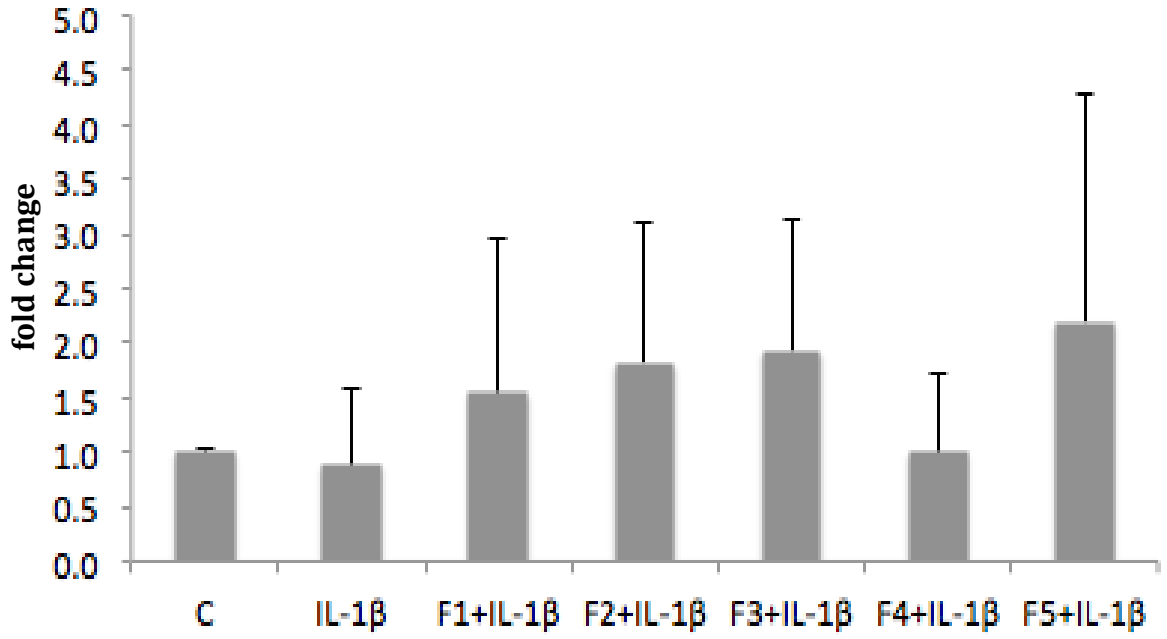

Figure 3. 14. HMOX1 mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of OA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 15. indicates HMOX1 levels under IL-1β presence and after pre-treatment with fractions of CSE in OA synoviocytes of the same patient introduced in Figure 3. 13.. Again it is striking to see, as in Figure 3. 14., that HMOX1 levels stay unaffected under IL-1β presence, whereas brazilin showed a remarkably high up-regulation of HMOX1 gene expression rates with 44.82±14.6-fold (p=0.06). The other fractions were not able to show statistical significance, as compared to the control.

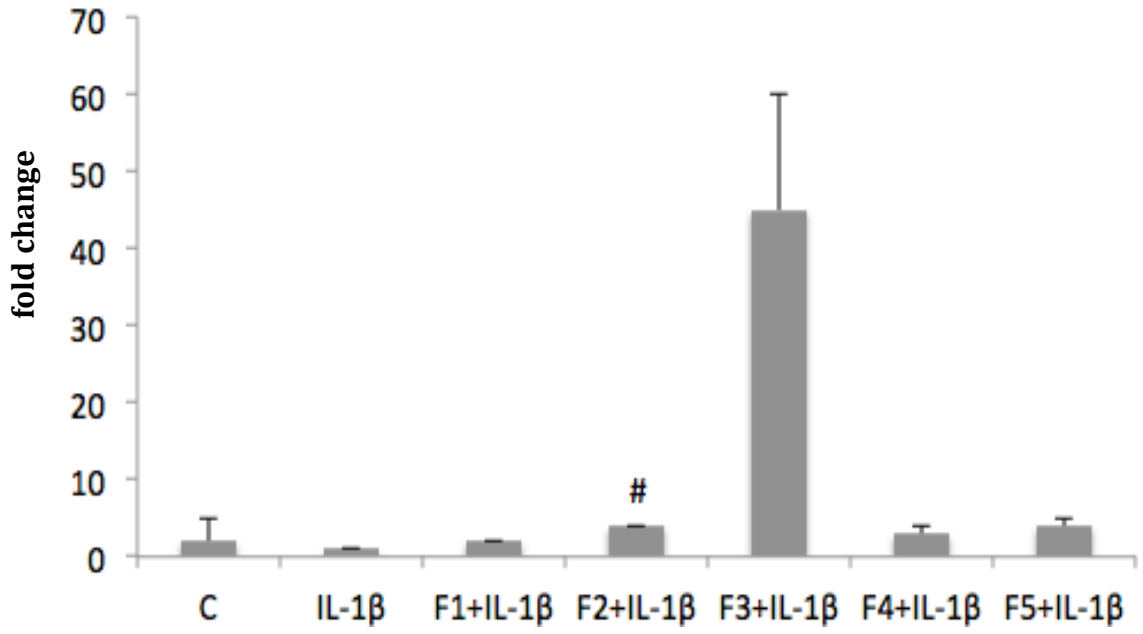

Figure 3. 15. HMOX1 mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the cytokine control, rhombus mark a statistically significant change of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 16. displays the regulation of IL1B mRNA levels in OA synoviocytes under CSE treatment with CSE fractions. The range of IL1B gene expression levels extends from a 1.01±0.53-fold (F2) to a 2.33±2.1-fold (F4) modification. Within this range, fraction four showed the highest up-regulation of IL1B mRNA levels with 2.33±2.1-fold, compared to the control. Overall, no significant results were reached due to inter-individual variability.

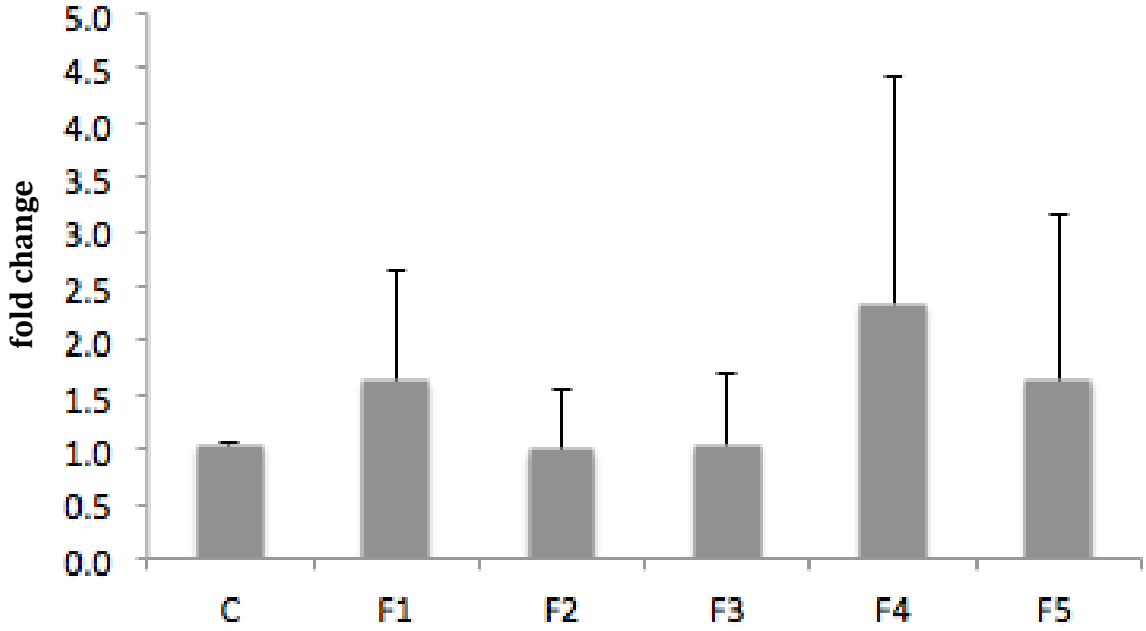

Figure 3. 16. IL1B mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of OA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

In Figure 3. 17., results of qPCR are shown regarding the capacity of CSE fractions to regulate IL1B gene expression levels. A minimum mRNA modification of 0.75±0.18-fold (F1) and a maximal change of 4.08±4.73-fold (F3) were observed. In summary, there was no significance for all five fractions (F1-F5) in direct comparison to the untreated control.

The results of this qPCR included only one patient, the same as in Figure 3. 13. and 3. 15., because the data did not fit into the preceding qPCR series, as seen in Figure 3. 16. considering a number of three patients.

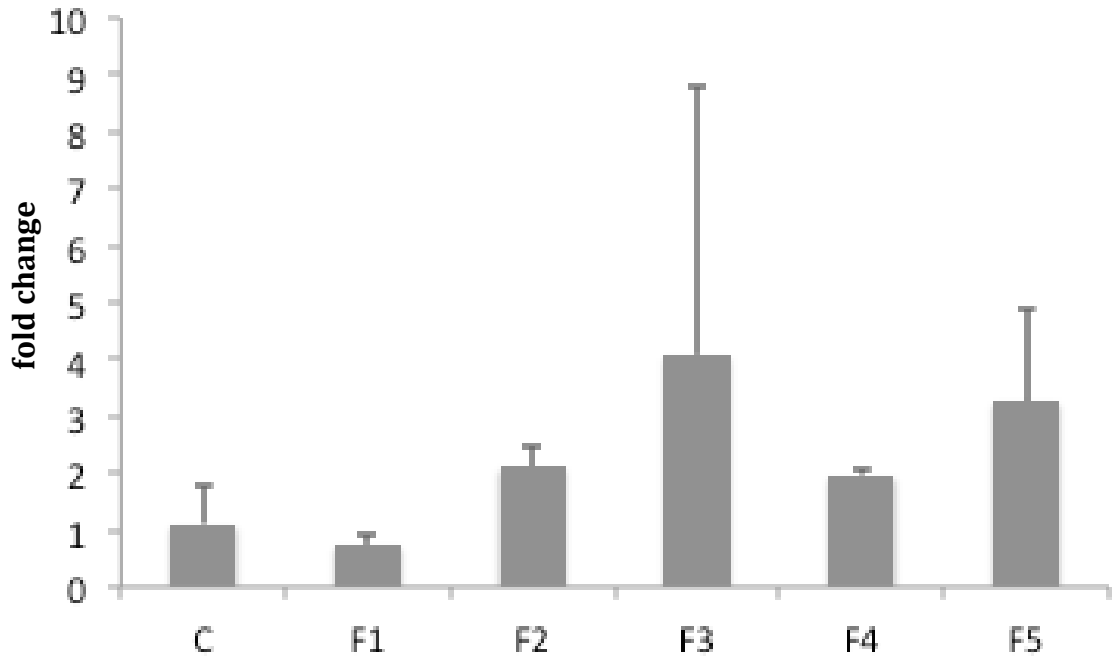

Figure 3. 17. IL1B mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

The modification of IL1B expression levels following additional IL-1β stimulation and pre-treatment with 5 µg/ml of CSE fractions is shown in Figure 3. 18..

Levels of IL1B mRNA were altered in a range from 1,014±1,295-fold (F4+IL-1β) to 2,619±2,273-fold (F3+IL-1β). In this experiment, IL-1β alone induced a 1,732±1,452 fold up-regulation as compared to the untreated control. In comparison to the control, brazilin (F3) increased the expression levels 2,619±2,273-fold in presence of IL-1β. This increase was higher than that of IL-1β, yet this result reached no statistical significance in direct comparison to the control. Also fraction five, with a raise of 2,266±1,949-fold and fraction two with 2,256±1,902-fold raise, compared to the control, showed a higher increase of gene expression levels than IL-1β. In contrast, only fraction four decreased the IL1B level in presence of the same cytokine, in comparison to the cytokine control. However, across all patients there was no significance reached due to inter-individual differences, in comparison to IL-1β as well as to the untreated OA synoviocytes as control group.

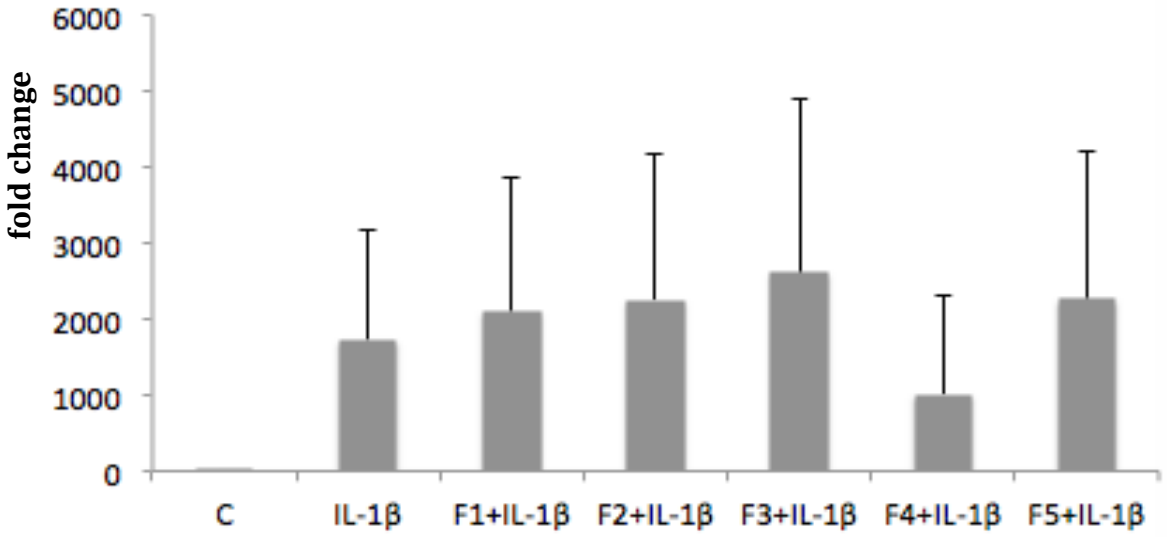

Figure 3. 18. IL1B mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of OA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Also in this experimental series, it was obvious that the one patient did not fit into the resulting outcomes, as shown in Figure 3. 19..

In presence of IL-1β, the range of altered IL1B expression levels starts from a minimum of 20.24±2.36-fold (F3+IL-1β) and shows a maximum of 3,635±293.6-fold (F5+IL-1β). Considering only the interval between the minimal and maximal increase in comparison to the control a much greater range is visible in comparison to the range of Figure 3. 18.. An IL-1β stimulation of OA synoviocytes led to a significant 1,772±153.8-fold raise of IL1B mRNA levels in direct comparison to the untreated control. In contrast to this increase, an additional pre-treatment with brazilin led to basal levels of IL1B gene expression. As such brazilin was able to induce a statistical significance of p=0.01, in comparison to IL-1β. This result is in contrast to the qPCR experiments illustrated in Figure 3. 18., which showed for brazilin a 2,619±2,273-fold raise of IL1B levels, compared to the control. In contrast, fraction one, two, four and five induced significantly higher IL1B mRNA levels (3,071±2.28, 3,210±50.08, 2,419±102.4 and 3,635±293.6-fold respectively), in comparison to IL-1β.

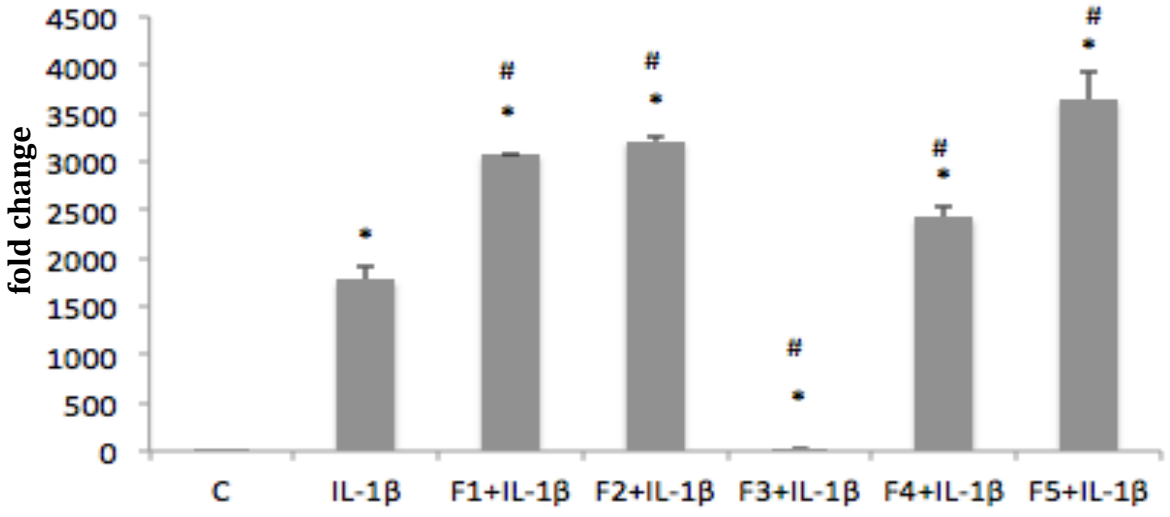

Figure 3. 19. IL1B mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistical significant change of gene expression levels, in comparison to IL-1β they were indicated by rhombus. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 20. reveals the regulation of MMP13 gene expression levels following a 5 µg/ml treatment with CSE fractions. Within a minor range from 0.40±0.37-fold (F3) to 0.96±0.47-fold (F1) alteration, compared to the untreated control, no significant results were reached because of inter-individual variability between the patients. In direct comparison to the untreated control, all five fractions led to similar basal levels of MMP13 mRNA.

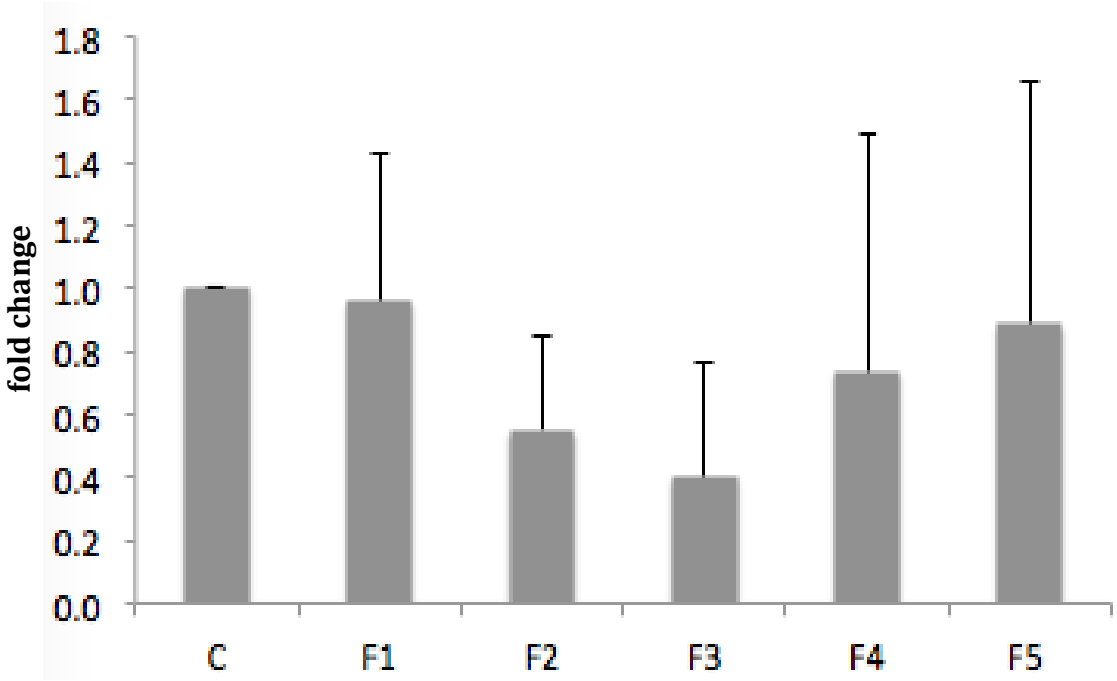

Figure 3. 20. MMP13 mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of OA patients (n=2) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 21. displays the influence of CSE fractions on mRNA levels of MMP13 in OA synoviocytes of the same patient as in previous experimental series of OA synoviocytes with HMOX1 and IL1B mRNA levels (→Figure 3. 13., 3. 15., 3. 17., 3. 19.).

Also in the MMP13 qPCR series, greater variability was observable in this patient. In this qPCR, no significant differences were visible. In direct comparison to the untreated control, brazilin (F3) led to a not significant 1.4±0.61-fold up-regulation. Likewise, fraction one induced a non-significant change of 0.05±0.01-fold, fraction four of 0.17±0.02-fold and fraction five of 0.05±0.06-fold.

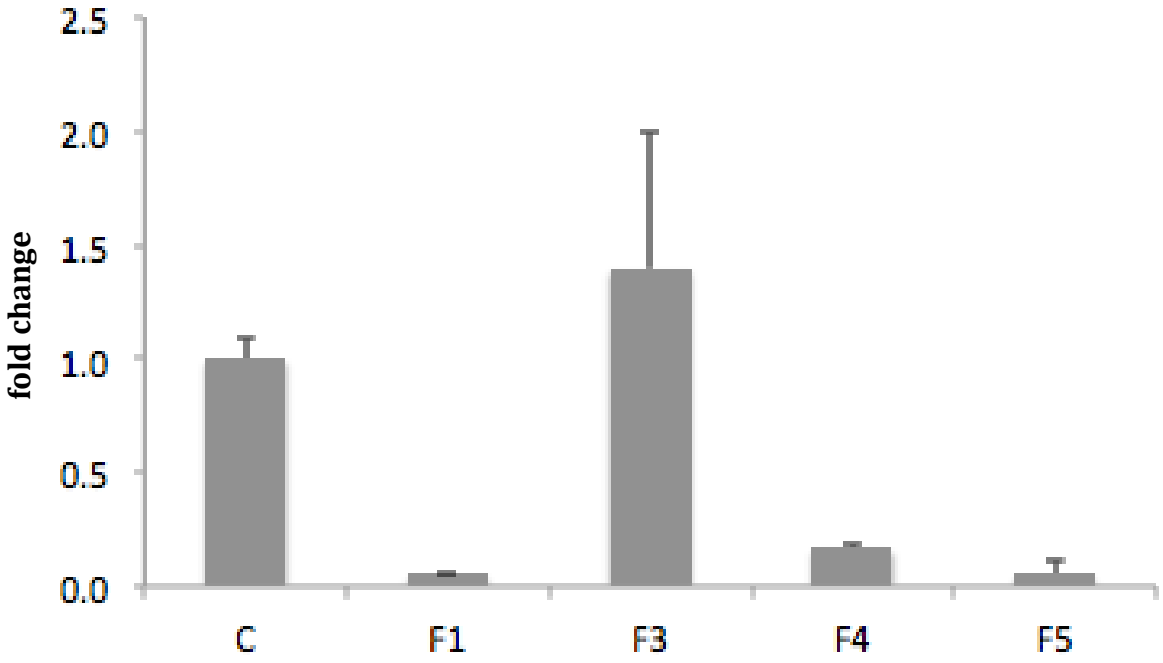

Figure 3. 21. MMP13 mRNA levels of OA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated OA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

To analyze the regulative capacity of CSE fractions under inflammatory conditions, further qPCR experiments were performed with an additional IL-1β stimulation, as shown in Figure 3. 22.. An alteration of MMP13 was observable in a range from 51.49±49.34-fold (F3+IL-1β) to 153.4±190.6-fold (F1+IL-1β). In direct comparison to the untreated synovial control, IL-1β showed a non-significant 150±118.8-fold increase. Fraction two and fraction three were able to decrease the MMP13 expression levels, in comparison to IL-1β with a change of 60±44.05-fold and 51.49±49.34-fold. Also fraction four, with 104.3±94.14-fold increase and fraction five with an increase of 98.5±97.15-fold, compared to the control, down-regulated the gene levels in direct comparison to IL-1β. In contrast, fraction one led to a change of 153±190.6-fold of MMP13, compared to the control. However, no significance was reached across all patients due to inter-individual differences.

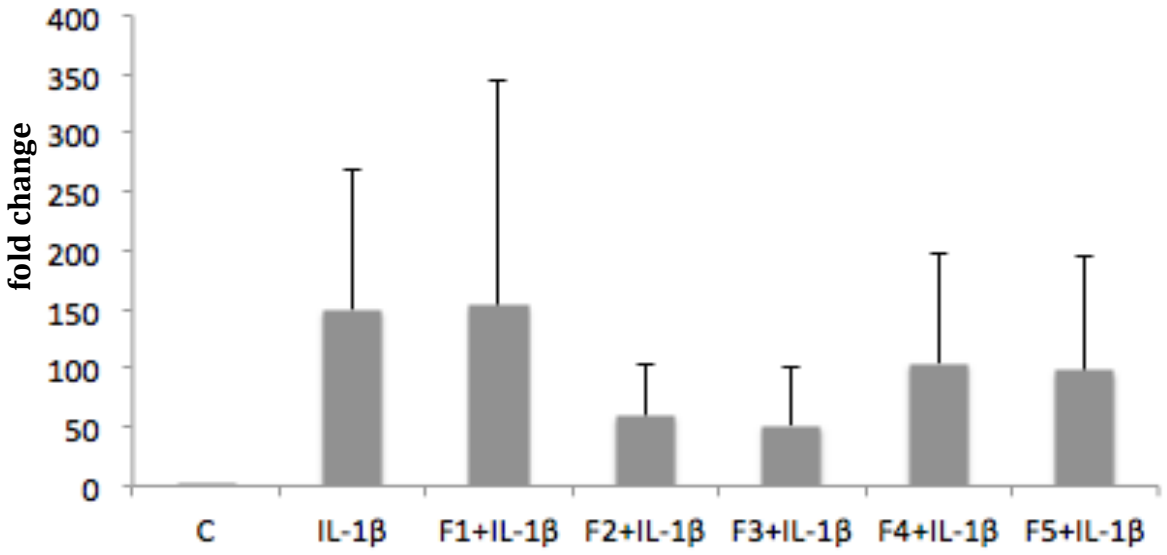

Figure 3. 22. MMP13 mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of OA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 23. indicates the MMP13 mRNA expression levels of one patient. Also in the MMP13 qPCR series, under IL-1β presence, greater variability was observable in OA synoviocytes of the same patient as in previous experimental series of OA synoviocytes with HMOX1 and IL1B mRNA levels (→Figure 3. 13., 3. 15., 3. 17., 3. 19.). The gene expression level was regulated within a range from 0.23±0.32-fold (F3+IL-1β) to 10.29±1.72-fold (F5+IL-1β). IL-1β significantly up-regulated MMP13 mRNA levels 16.18±3.75-fold in direct comparison to the untreated control. Brazilin significantly decreased MMP13 gene expression levels in comparison to IL-1β. With a change of 0.23±0.32-fold, compared to the control, basal levels were reached following pre-treatment with brazilin. Fraction one, two and four significantly reduced MMP13 levels in comparison to IL-1β. A statistical significance, in direct comparison to the control, was reached for the fractions one, two, four and five.

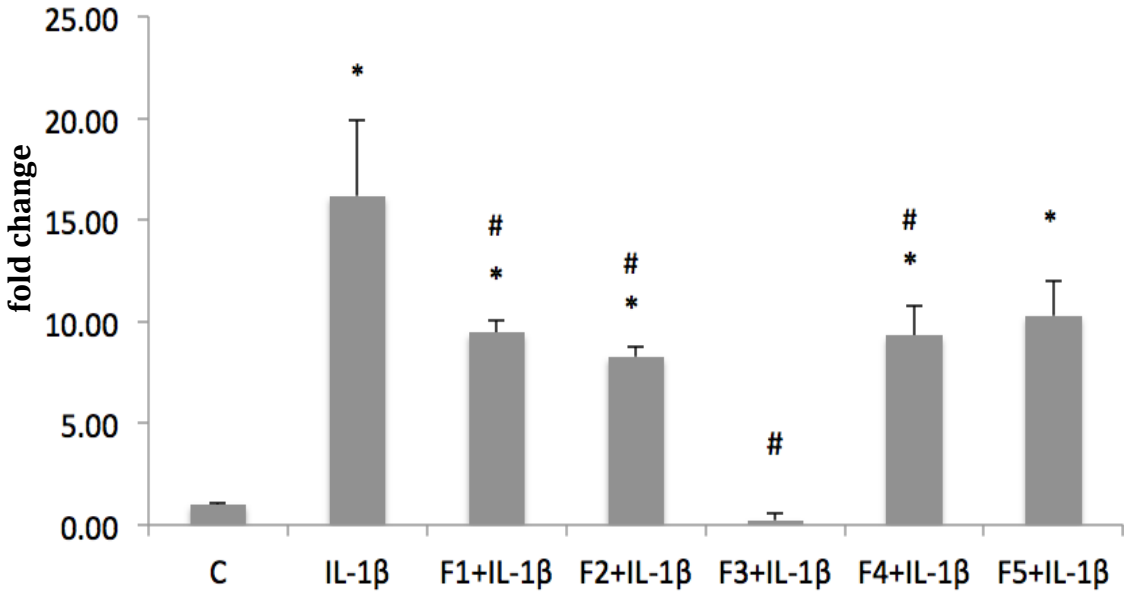

Figure 3. 23. MMP13 mRNA levels of OA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of an OA patient (n=1) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated OA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant change of gene expression levels, in comparison to IL-1β they were indicated by rhombus. P-values < 0.05 were considered significant (determined by t-test).

3.4.3. mRNA levels of RA synoviocytes

The gene expression pattern of OA chondrocytes was not reproducible in OA synoviocytes as seen in the figures of → 3.4.2. mRNA levels of OA synoviocytes. For yet undetermined reasons, one patient of the OA synoviocytes series fell out of the qPCR results and showed gene expression patterns comparable to OA chondrocytes. The resulting question was if these findings correspond to a basic inflammatory status, which is the reason why further qPCR were performed on RA synoviocytes.

Figure 3. 24. displays the HMOX1 mRNA levels after treatment with CSE fractions in RA synoviocytes. The increase of gene expression levels showed an interval from 1.46±0.83-fold (F1) change to 37.09±21.58-fold (F3) compared to the control. In direct comparison to the control, fraction one with 1.46±0.83-fold change, fraction two with 2.02±0.1-fold change, fraction four with 1.62±0.46-fold change and fraction five, with 2.51±0.82-fold change, showed no significant alterations of HMOX1 levels. In contrast, brazilin achieved a remarkable 37.09±21.58-fold increase compared to the untreated control. However, across all patients no statistical significance was reached due to inter-individual variability.

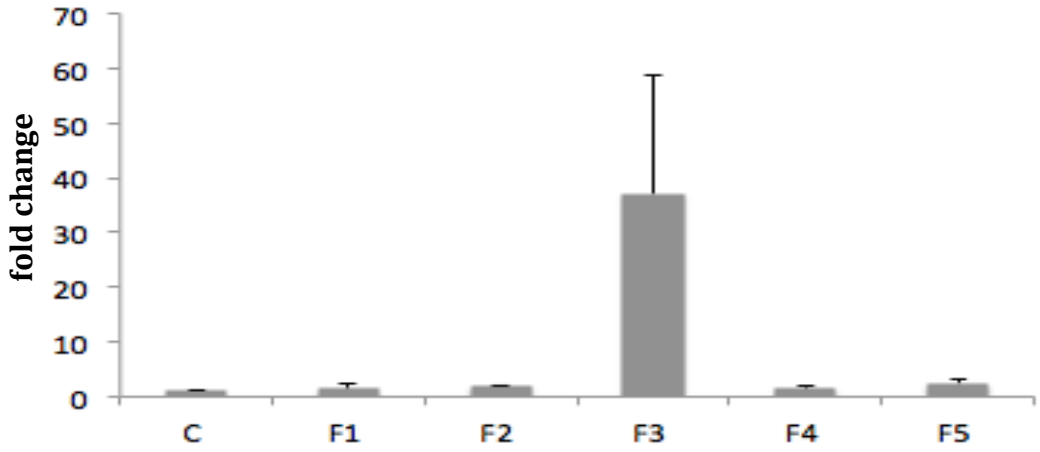

Figure 3. 24. HMOX1 mRNA levels of RA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated RA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

The results of Figure 3. 24. were solidified by the following Figure 3. 25., which shows the influence of fractions on HMOX1 expression levels under an additional cytokine stimulation. It is observable that cells treated only with IL-1β showed no significant up-modulations, as compared to the untreated control. Also fraction one (1.06±0.85-fold), fraction two (0.54±0.09-fold), fraction four (1.23±1.17-fold) and fraction five (0.35±0.09-fold), did not induce a significant increase as compared to the control in presence of IL-1β. In contrast, brazilin (F3) led to a 24.62±1.08-fold up-regulation of HMOX1 levels as compared to the control. This result reached statistical significance as compared to the untreated control as well as to IL-1β.

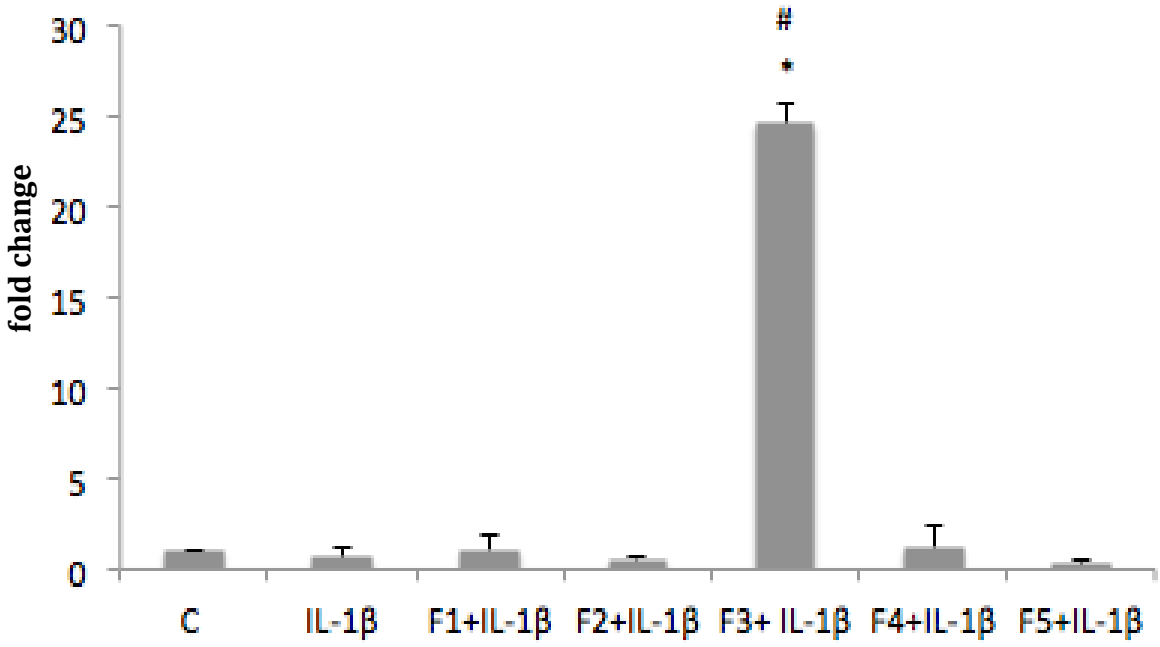

Figure 3. 25. HMOX1 mRNA levels of RA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated RA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant change of gene expression levels, in comparison to IL-1β they were indicated by rhombus. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 26. illustrates the regulation of IL1B mRNA levels. Comparable to the untreated RA synoviocytes, all five fractions showed only slight up-regulations of IL1B mRNA levels. This modification ranged from 2.27±1.26-fold (F4) to 5.84±3.28-fold (F5). As compared to the control, the maximal raise of 5.84±3.28-fold was reached by fraction five. Across all patients, however, there was no significance reached because of inter-individual differences.

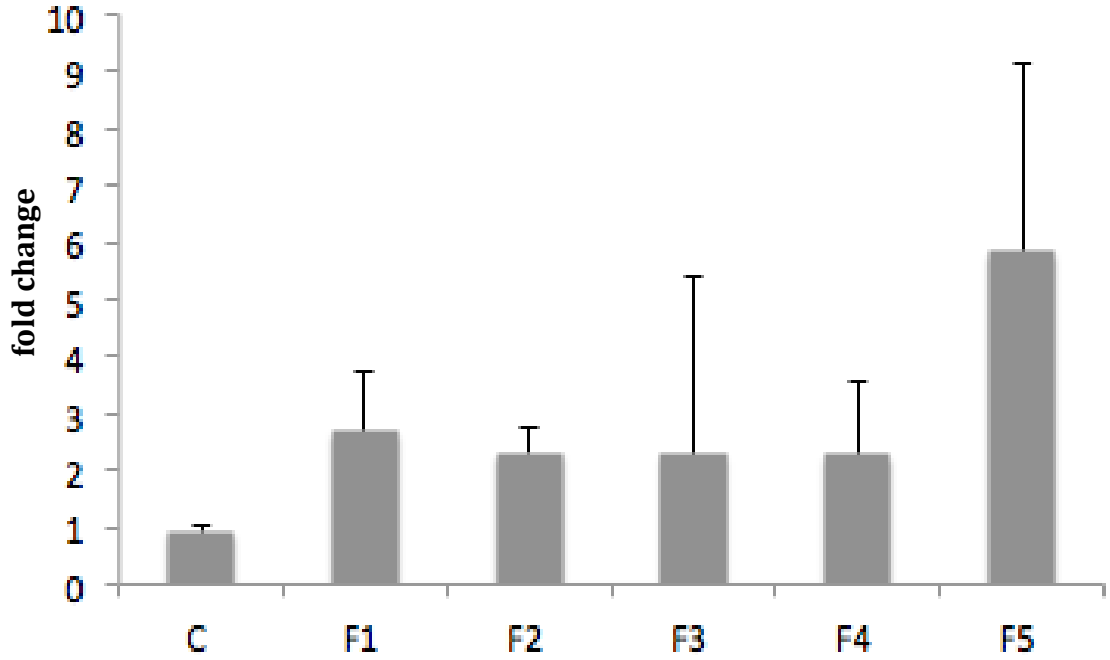

Figure 3. 26. IL1B mRNA levels of RA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated RA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No significance was reached, p-values < 0.05 were considered significant (determined by t-test).

In Figure 3. 27. the influence of CSE fractions on IL1B gene expression levels in presence of IL-1β is displayed. Gene expression levels were altered in a range from 25.57±34.28-fold (F3) to 1290±1506-fold (F1), as compared to the control. IL-1β induced a 770.9±949-fold increase of IL1B levels as compared to the untreated control. In direct comparison to the untreated control, brazilin (F3) altered IL1B mRNA levels 25.57±34.28-fold. This reduction of IL1B by brazilin as compared to the IL-1β treated control is in line with the results of previous HMOX1 qPCR experiments with RA synoviocytes (→Figure 3. 26.).

Across all patients, no statistical significance could be reached due to inter individual-variability.

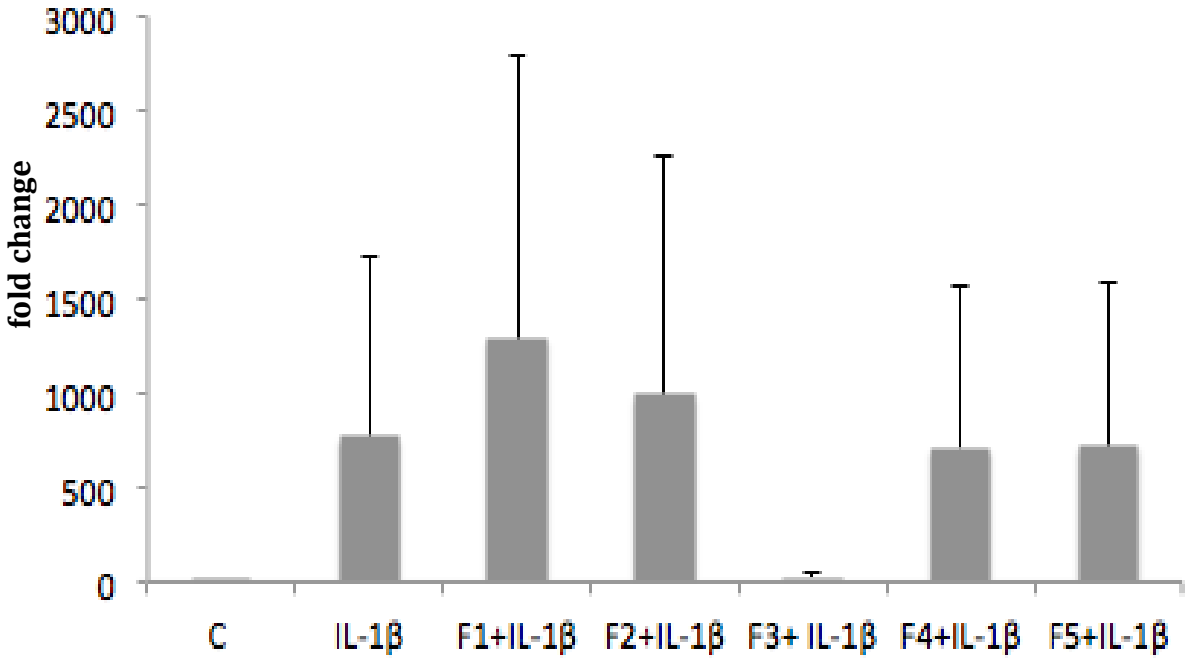

Figure 3. 27. IL1B mRNA levels of RA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated RA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of IL1B, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

Figure 3. 28. indicates MMP13 gene expression levels following treatment with 5 µg/ml of CSE fractions. mRNA levels were altered from 0.97±0.09-fold (F3) to 2.3±1.47-fold (F5). Across all patients no statistical significance was reached, in comparison to the control, due to inter-individual differences.

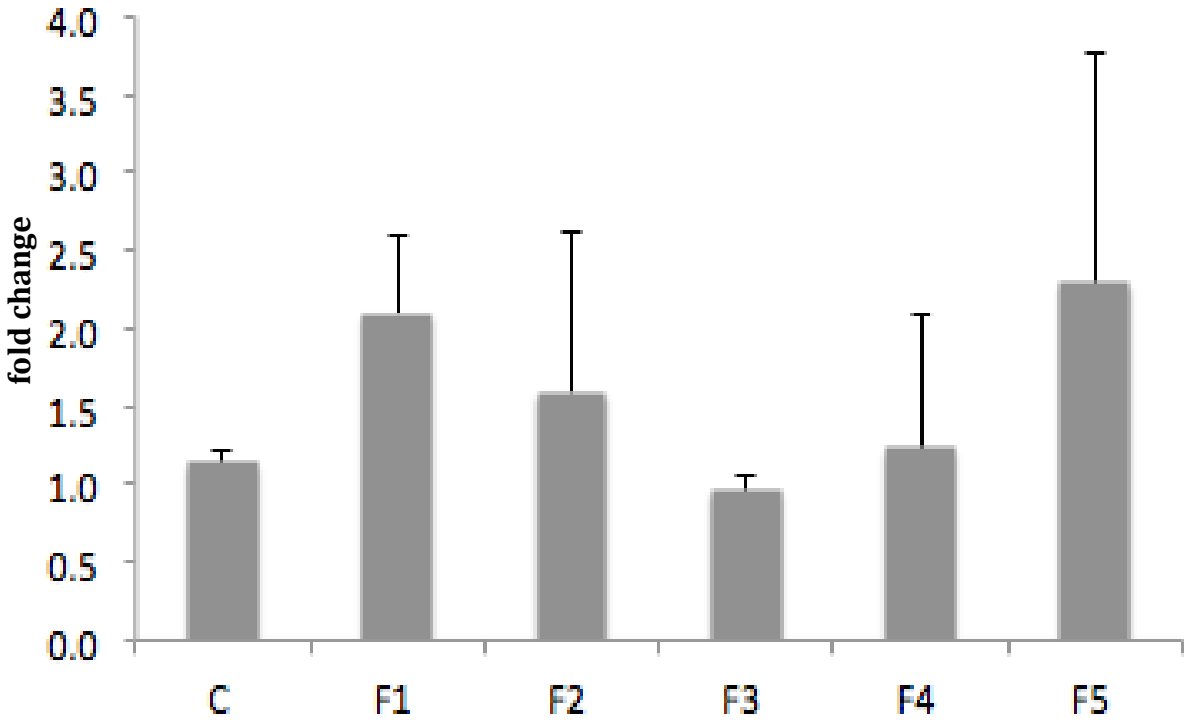

Figure 3. 28. MMP13 mRNA levels of RA synoviocytes after treatment with CSE fractions

An overnight treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated RA synoviocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

The alteration of MMP13 mRNA levels in presence of IL-1β and following pre-treatment with fractions of CSE, are displayed in Figure 3. 29.. Expression levels of MMP13 were changed in a range from 109.1±153.5-fold (F3+IL-1β) to 330±424.4-fold (F1+IL-1β). In direct comparison to the untreated RA synoviocytes, a treatment with only IL-1β led to an increase of 213.3±259.2-fold of MMP13 levels. Consequently, MMP13 gene expression levels were increased under inflammatory conditions. In comparison to IL-1β, brazilin led to a reduction of cytokine expression levels in IL-1β presence, with an observed modification of 109.1±153.5-fold as compared to the control. However, due to inter-individual differences no statistical significance was reached across all patients.

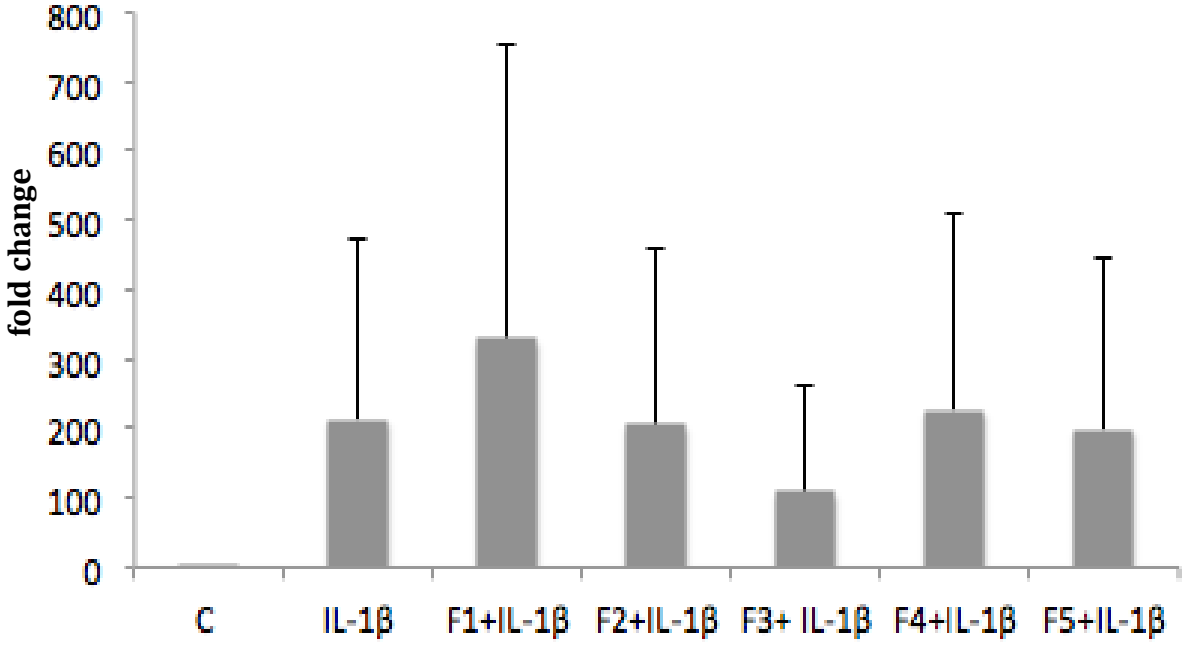

Figure 3. 29. MMP13 mRNA levels of RA synoviocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on synoviocytes of RA patients (n=3) with 5 µg/ml of five different fractions (F1-5) of CSE, followed by an overnight co-incubation with 10 ng/ml IL-1β. As reference, untreated RA synoviocytes (C) and synoviocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. No statistical significance was reached, p-values < 0.05 were considered significant (determined by t-test).

3.4.4. mRNA levels of non-OA chondrocytes

To investigate anti-arthritic effects of CSE fractions, several qPCR experiments were performed in OA chondrocytes, OA synoviocytes and on RA synoviocytes. To analyze if these inhibiting properties of CSE components are dependent on the arthritic disease status, further experiments using non-OA chondrocytes were performed.

Figure 3. 30. displays the influence of the isolated compounds on HMOX1 mRNA expression levels in non-OA chondrocytes. The modification of HMOX1 gene expression levels showed a minimum of 1.307±0.125-fold (F2) and a maximum of 21.35±0.179-fold (F3). Within this range, brazilin (F3) induced a significant 21.35±0.179-fold increase of HMOX1mRNA levels in comparison to the control. In comparison fraction one, four and five showed a slight but significant 1.49±0.04-fold and 1.857±0.08-fold modification, respectively. Not significant was the 1.307±0.125-fold change of fraction two, as compared to the control.

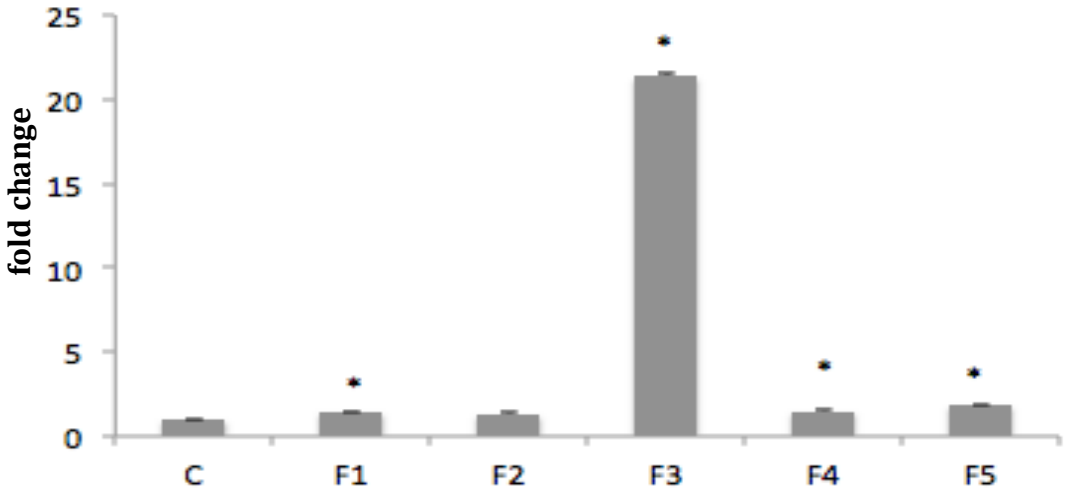

Figure 3. 30. HMOX1 mRNA levels of non-OA chondrocytes after treatment with CSE fractions

A twenty-four hour treatment was performed on chondrocytes of one non-OA patient (n=1) with 10 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated non-OA chondrocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant up-regulation of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

Figure 3. 31. extends the outcome of Figure 3. 30., as it illustrates the alterations of HMOX1 gene expression levels in non-OA chondrocytes treated with CSE fractions in presence of IL-1β. It is clearly observable that brazilin led to a 18.22±1.664-fold increase of HMOX1 levels in comparison to the untreated control. This result was statistically significant, compared to the control as well as to IL-1β-treated cells. In contrast, cytokine treatment with IL-1β led to non-significant modifications of HMOX1 gene expression levels. Comparable to the control, IL-1β showed a 1.692±0.072-fold alteration. Compared to IL-1β, fraction one, two, three, four and five led to non-significant changes of HMOX1 expression levels. The reached levels were all at basal levels, comparable to the untreated control.

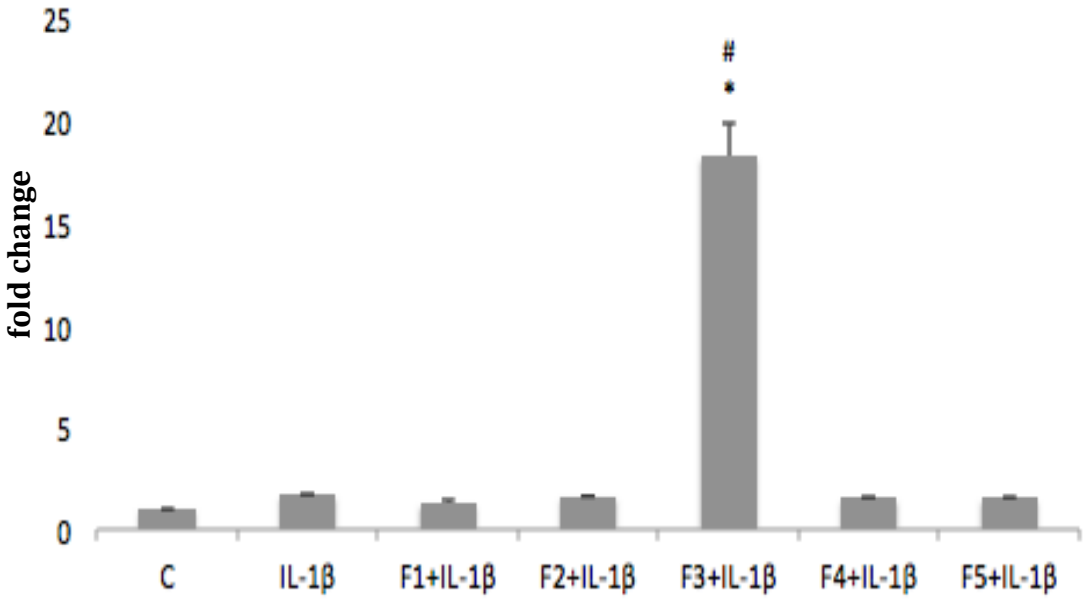

Figure 3. 31. HMOX1 mRNA levels of non-OA chondrocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed on chondrocytes of a non-OA patient (n=1) with 10 µg/ml of five different fractions (F1-5) of CSE, followed by a twenty-four hour co-incubation with 10 ng/ml IL-1β. As reference, untreated non-OA chondrocytes (C) and chondrocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of HMOX1, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant change of gene expression levels, in comparison to IL-1β they were indicated by rhombus. P-values < 0.05 were considered significant (determined by t-test).

Regarding the regulation of IL1B qPCR experiments were also performed with non-OA chondrocytes. Changes in IL1B expression levels were analyzed following a 10 µg/ml treatment with the five different fractions of CSE for 24 h. As control, untreated cells were used. Additionally, the alterations were analyzed following a cytokine co-incubation for 24 h with a 1 h pre-treatment with CSE fractions. Again, untreated non-OA chondrocytes were used as comparison, as were IL-1β treated cells.
All five components had no effect on IL1B expression levels, in presence and absence of IL-1β (data not shown).

Figure 3. 32. illustrates mRNA levels of MMP13 following a 24 h treatment with CSE fractions. The alterations of gene expression levels showed a minimum change of 0.326±0.008-fold (F3) and a maximal change of 1.564±0.094-fold (F5). Within this range, brazilin (F3) led to a significant decrease of MMP13 levels (0.326±0.008-fold). Fraction one (0.885±0.003-fold) and fraction two (0.78±0.222-fold) showed slight and non-significant decreases compared to the untreated control. In contrast, a significant 1.564 ±0.094-fold change of MMP13 gene expression levels were cause by fraction five.

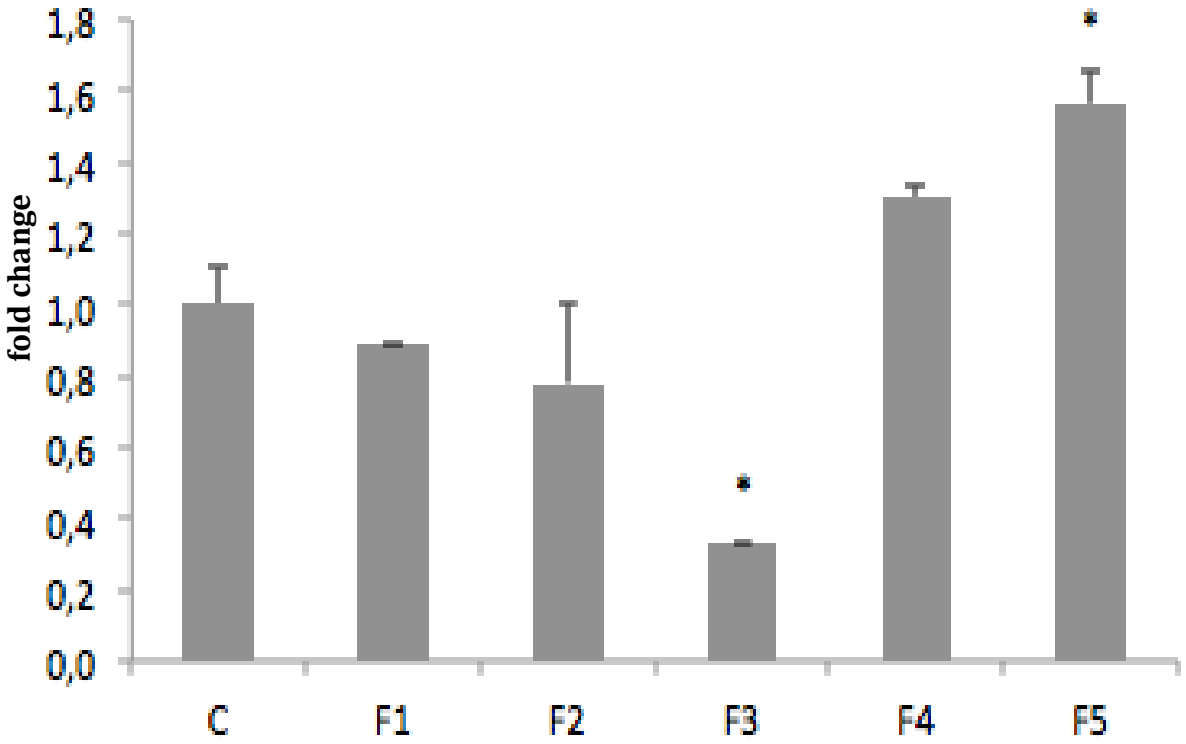

Figure 3. 32. MMP13 mRNA levels of non-OA chondrocytes after treatment with CSE fractions

A twenty-four hour treatment was performed on chondrocytes of a non OA patient (n=1) with 10 µg/ml of five different fractions (F1-5) of CSE. As reference, untreated non-OA chondrocytes (C) were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant change of gene expression levels. P-values < 0.05 were considered significant (determined by t-test).

In Figure 3. 33. MMP13 expression levels are shown following a pre-treatment with fractions of CSE and additional IL-1β stimulation. MMP13 mRNA levels were increased 1.683±0.174-fold by IL-1β, in comparison to the untreated control. Compared to IL-1β, brazilin (F3) significantly reduced MMP13 expression to 0.2163±0.031-fold levels, i.e. even below the untreated control. Significant, in direct comparison to IL-1β, was also the 1.033±0.022-fold change of fraction two.

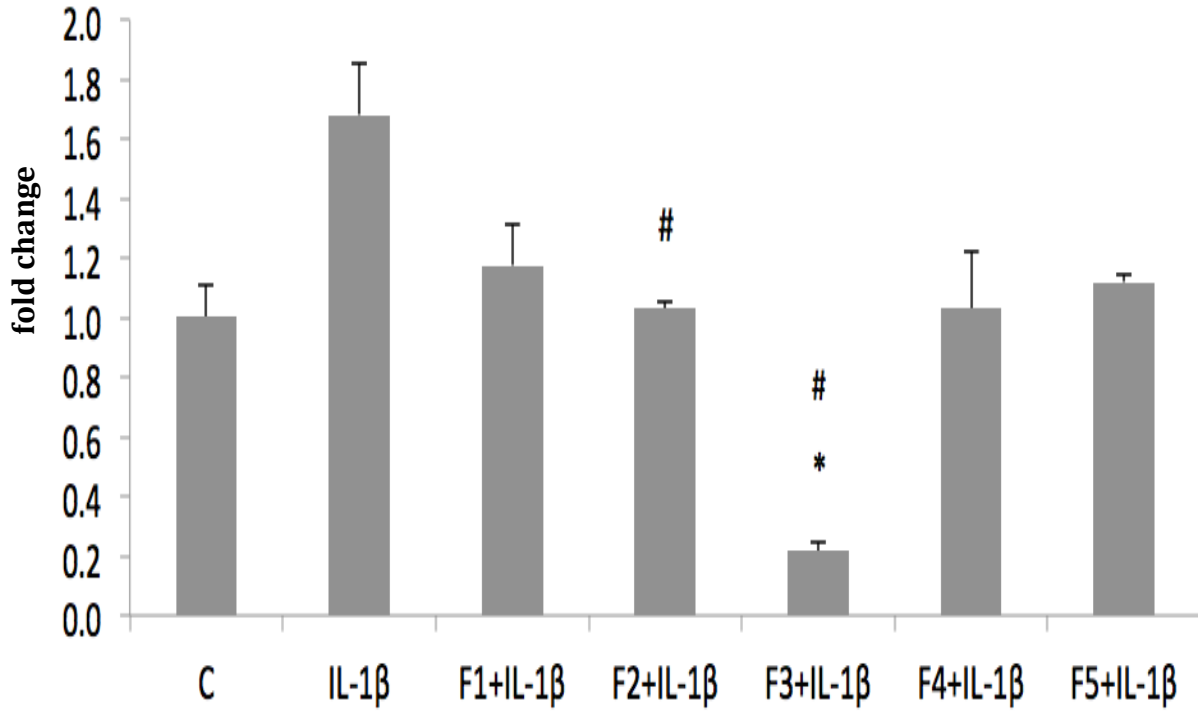

Figure 3. 33. MMP13 mRNA levels of non-OA chondrocytes after pre-treatment with CSE fractions and subsequent IL-1β-stimulation

One hour pre-treatment was performed with chondrocytes of a non-OA patient (n=1) with 10 µg/ml of five different fractions (F1-5) of CSE, followed by a twenty-four hour co-incubation with 10 ng/ml IL-1β. As reference, untreated non-OA chondrocytes (C) and chondrocytes treated only with IL-1β were cultivated under the same conditions. To quantify the raise of mRNA levels of MMP13, qPCR was performed relatively to SDHA as housekeeping gene. In comparison to the control, asterisks mark a statistically significant change of gene expression levels, in comparison to IL-1β they were indicated by rhombus. P-values < 0.05 were considered significant (determined by t-test).

3.5. Protein levels in brazilin treated cells

Western blot analysis was performed in OA chondrocytes to provide insight into the signaling pathways stimulated by brazilin, i.e. fraction three of CSE. OA chondrocytes (n=4) were pre-treated for one hour with 5 µg/ml brazilin and then stimulated with 10 ng/ml IL-1β for twenty-four hours. The aim of the Western blot experiments was to evaluate if regulated gene expression levels after brazilin treatment were also visible at protein levels. A clear increase of HMOX1 protein was observable, as shown in Figure 3. 34.. Consequently, Western blot analysis successfully supported previous qPCR results, regarding HMOX1.

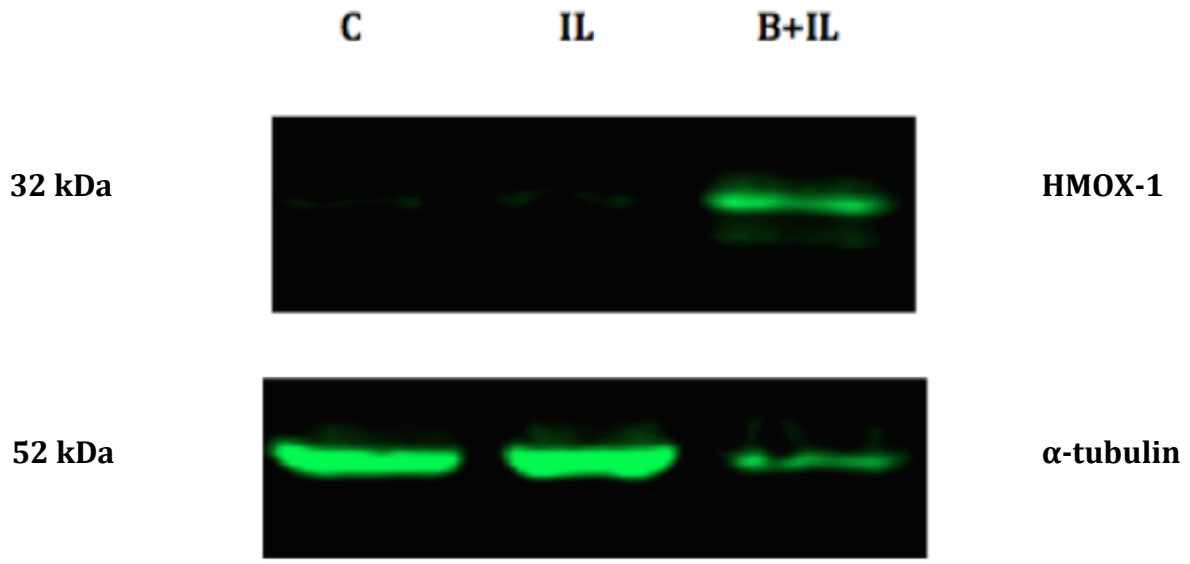

Figure 3. 34. HMOX-1 concentrations after a brazilin pre-treatment

Representatively shown is one Western blot of OA chondrocytes (n=4). The upper bands display the protein amounts of HMOX-1, whereas the lower bands show the reference protein α-tubulin. Cells were pre-treated with brazilin for one hour with 5 µg/ml and afterwards stimulated with 10 ng/ml IL-1β (B+IL) or were treated only with IL-1β (IL) or stayed untreated as control (C).

4. Discussion

Extracts of Caesalpinia sappan heartwood are used in the traditional Chinese medicine, because of its anti-inflammatory, analgesic and blood circulation improving benefits. After it had been proven that CSE was able to decrease IL-1β-induced mRNA levels of cytokines and MMPs (Toegel et al., 2012; Wu et al., 2011), a recent study provided evidence that brazilin inhibited the disease symptoms in a type-II collagen stimulated mouse models of RA (Jung et al., 2015). Since brazilin is the major representative among the main five components of CSE, this thesis aimed to test our hypothesis that brazilin is responsible for the beneficial effects of CSE in human arthritic joint cells. Additionally, we aimed to evaluate a correlation between brazilin activity and increased HMOX1 mRNA levels, in order to approve literature demonstrating the protective influence of brazilin on chondrocytes (Clérigues et al., 2013) and brazilin-mediated induction of HMOX1 gene expression rates in RAW264.7 macrophages (Hu et al., 2009).

First, we isolated the five main active substances of CSE performing preparative HPLC, and confirmed their purity for further biological assays applying analytical HPLC. The five components of the plant material were identified as episappanol, protosappanin C, brazilin, protosappanin B and sappanol. Brazilin appears to be the component of the highest interest, because it is the major component and its activity has already been addressed in many studies. This major component has a defined pure chemical structure with an IUPAC denotation and a CAS-number, but sill the name brazilin is used not only for this chemical component. This causes confusion because commercially available Natural Red 24, which is used industrially to produce dyes sand inks, is also known under “brazilin”. In contrast to the defined chemical substance brazilin, “brazilin”/Natural Red 24consist of various not defined components. However, for biological assays it is necessary to define used substances in quantitative and qualitative terms, only in this way biological activities can be identified. But still this important issue has not been attached sufficient importance in the literature (Bae et al., 2005; Lee et al., 2012; Chang et al., 2013). Consequently, we isolated brazilin of plant material and ensured its purity performing preparative and analytical HPLC, respectively.

Prior to testing the biological effects of CSE components, we determined the range of non-toxic concentrations of the components in patient-derived cell populations. Therefore, the impact of each component on the metabolic cell activity was analyzed at different concentrations in chondrocytes and synoviocytes, as an indicator for cell viability. Resulting data allowed us to perform further treatments at non-toxic concentrations of each component.

Regarding the cellular events in arthritic dieseases, inflammation has been recognized as a major contributing factor for the progression of joint destruction. Intracellular stress factors drive a cascade of signals that eventually lead to the breakdown of cartilage tissue. This process is characterized by increased gene expression levels of catabolic cytokines, such as IL-1β and TNFα, which further increase mRNA levels of matrix-degenerating MMP13 (Goldring et al., 2009). Based on these facts, we analyzed in our study the gene expression levels of IL1B and MMP13 in human arthritic chondrocytes as well as in human arthritic synoviocytes. Also, we set a focus on the protective enzyme HMOX1, since its protective effects on chondrocytes had already been demonstrated (Clérigues et al., 2013).

Gene expression levels were quantified performing RT-qPCR. Here, we show that brazilin induces a down-regulation of IL-1β-stimulated over-expression of pro-inflammatory IL1B and matrix-degenerating MMP13 in OA chondrocytes. Both genes play a key role in the pathogenic degeneration of cartilage tissue within OA and RA. These results are in agreement with Toegel et al. (2012) and Wu et al. (2011), who provided evidence for an inhibition of IL-1β-mediated MMP13 and IL1B overexpression by CSE. The other four components, in contrast, showed no effects in the present study, further highlighting the important role of brazilin for CSE activity.
In agreement, only brazilin was able to increase HMOX1 mRNA levels in OA chondrocytes. This increase was achieved in presence and absence of the inflammatory cytokine IL-1β. Because the raise of HMOX1 mRNA levels was higher in IL-1β presence, we came to the assumption that the effect of brazilin might correlate with inflammatory conditions. The other four studied components, in contrast, did not show any effects on HMOX1 gene expression levels which remained at basal levels. We additionally solidified the results of raised HMOX1 gene expression levels in OA chondrocytes performing

Western blotting. Resulting data showed that also HMOX-1 protein levels were increased after brazilin treatment.

In contrast to OA chondrocytes, brazilin showed no impact on OA synoviocytes. This is interesting as García-Arnandis et al. (2010) had provided evidence that increased HMOX1 levels – induced by cobalt protoporphyrin IX – were able to reduce MMP1 and MMP3 mRNA levels in OA synoviocytes, showing that a relation between HMOX1 and MMP levels would generally exist in synoviocytes. The reason why synoviocytes were not responsive to brazilin in our study still remains to be explored. However, for yet undetermined reasons one cell population of the OA synoviocytes group behaved differently, showing similar gene expression patterns as observed in OA chondrocytes. As such, IL-1β-induced overexpression of IL1B and MMP13 was reduced after brazilin treatment in this one cell population. In agreement, HMOX1 mRNA levels were raised at the same time, supporting the findings by García-Arnandis et al. (2010) who provided evidence for a relation between HMOX1 and MMP levels. Interestingly, the only main difference of this patient, in his medical history, was an alcohol abuse. Since previous studies showed an up-regulation of inflammatory markers in patients with alcohol dependency (Fuster et al., 2015), this underscores our suggestion that the effect of brazilin might correlate with inflammatory conditions. However, it would be of high interest to consider this fact in further investigations.

Following this line of argumentation, HMOX1 gene expression was further analyzed in RA synoviocytes. Compared to OA synoviocytes, RA synoviocytes – due to the different pathogenesis of RA – provide higher rates of inflammation. Here, we again observed an increase of HMOX1 mRNA levels following brazilin stimulation, in presence and absence of IL-1β. In agreement with this finding, brazilin suppressed the levels of IL-1β-induced overexpression of IL1B and MMP13. This is in agreement with literature presenting evidence for a relation between HMOX1 and MMP levels (García-Arnandis et al., 2010) and underscores our assumption that brazilin effects are dependent on an inflammatory status, because impact of brazilin were observable in RA but not in OA synoviocytes.

Interestingly, HMOX1 mRNA levels were also induced by brazilin in non-OA chondrocytes – both in presence and absence of IL-1β –, followed by a reduction of MMP13 levels. Consequently, our results are in line with V. Clérigues et al. (2013), who

showed that IL-1β inhibits HMOX1 mRNA levels in OA and healthy chondrocytes. This inhibition led to catabolic results and was able to be reversed by a cobalt protoporphyrin IX-induced raise of HMOX1 gene expression rates. Also, there is an agreement with I. Devesa et al. (2005) who illustrated that protoporphyrin IX-induced HMOX1 mRNA levels suppressed the erosion of cartilage in murine models of collagen induces arthritis.

Taken together, our results on brazilin activity in OA chondrocytes and RA synoviocytes are in accordance with a study showing the effectively decreased inflammatory levels of IL-1β, IL-6 and TNFα in the type-II collagen-induced arthritis in mouse model, after a brazilin treatment (Jung et al., 2015). In the future, it would be of high interest to perform similar investigations in vivo using an animal model of OA to establish whether brazilin might be a potential therapeutic agent for osteoarthritic conditions.
As an outlook, clinical investigations addressing a possible application of brazilin in OA patients are also anticipated. Prior to a possible application of brazilin in humans, however, a range of preclinical problems will have to be solved. For instance, the chemical stability of brazilin will demand further attention, as will the issue of toxicity, bioavailability, dosage forms and route of administration. Recent studies showing the feasibility of a total chemical synthesis of brazilin might reduce the need for phytochemical extraction and might contribute to the development of pre-clinical and clinical studies (Wang et al., 2013; Jung et al., 2015).

5. Conclusion

The results of this thesis suggest that brazilin plays a key role in the anti-inflammatory activity of CSE. The main five components (episappanol, protosappanin C, brazilin, protosappanin B and sappanol) were isolated from the plant material to investigate their influence on HMOX1, IL1B, MMP13 gene expression levels and HMOX-1 protein expression rates. All five components were tested at non-toxic concentrations on human arthritic chondrocytes and synoviocytes.

In general, brazilin showed strong effects on gene expression rates. In OA chondrocytes, brazilin was able to decrease IL-1β-mediated up-regulation of MMP13 and IL1B mRNA levels. In agreement, it concurrently increased HMOX1 gene expression rates and HMOX-1 protein levels. Similarly, non-OA chondrocytes showed, after brazilin treatment, an increase of HMOX1 gene expression levels and a concurrent reduction of MMP13 mRNA levels. In contrast, brazilin did not show any impact in OA synoviocytes except for one patient in which gene expression patterns similar to those in OA chondrocytes were observed. In this patient, of note, a concurrent up-regulation of HMOX1 and down-regulation of MMP13 and IL1B mRNA levels was shown. The impact of brazilin was also evaluated in RA synoviocytes. Here, the IL-1β-mediated increase of IL1B and MMP13 mRNA levels was suppressed whereas HMOX1 mRNA levels were up-regulated by brazilin.

Taken together, this thesis suggests that brazilin might be responsible for the anti-inflammatory effect of CSE in the context of arthritic diseases. Furthermore, the results indicate that the activity of brazilin correlates with increased levels of HMOX1 gene expression levels.

6. References

6.1. Books

Allgemeine und spezielle Pharmakologie und Toxikologie - 10. Auflage; Urban und Fischer in Elsevier Verlag 2009. Aktories, Förstermann, Hofmann, Starke

Prometheus - Lernatlas der Anatomie, allgemeine Anatomie und Bewegungssystem, 4. Auflage; Georg Thieme Verlag 2014. Schünke, Schulte, Schumacher, Voll, Wesker.

6.2. Papers

Abolhassanzadeh Z, Aflaki E, Yousefi G, Mohagheghzadeh A. Randomized clinical trial of peganum oil for knee osteoarthritis. J Evid Based Complementary Altern Med 2015; 20:126-31.

Akhtar N, Haqqi T. Current nutraceuticals in the management of osteoarthritis: a review. Ther Adv Musculoskelet Dis 2012; 4:181–207.

Bae I, Min H, Han A, Seo E, Lee S. Suppression of lipopolysaccharide-induced expression of inducible nitric oxide synthease by brazilin in RAW 264.7 macrophage cells. Eur J Pharmacol 2005; 513:237-242.

Benito M, Veale D, FitzGerald O, van den Berg W, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2014; 64:1263-7.

Brix M, Chiari C. Knorpelregeneration. Z Orthop Unfall 2012; 150:1-23.

Chang Y, Huang S, Lu W, Chung C, Chen W, Lu S, Lin K, Sheu J. Brazilin isolated from Caesalpinia sappan L. acts as a novel collagen receptor agonist in human platelets. J Biomed Sci 2013; 20:4.

Clériques V, Murphy C, Guillén M, Alcaraz M. Haem oxygenase-1 induction reverse the actions of interleukin-1β on hypoxia-inducible transcription factors and human chondrocyte metabolism in hypoxia. Clin Sci (Lond) 2013; 125:99-108.

Devesa I, Ferrándiz M, Terencio M, Joosten L, van den Berg W, Alcaraz M. Influence of heme oxygenase 1 modulation on the progression of murine collagen-induced arthritis. Arthritis Rheum 2005; 52:3230-8.

Fidelix T, Macedo C, Maxwell L, Fernandes Moça Trevisani V. Diacerein for osteoarthritis. Cochrane Database Syst Rev 2014; 2:CD005117

García-Arnandis I, Guillén M, Castejón M, Gomar F, Alcaraz M. Haem oxygenase-1 down-regulates high mobility group box 1 and matrix metalloproteinases in osteoarthritic synoviocytes. Rheumatology (Oxford) 2010; 49:854-61.

Glyn-Jones S, Palmer A, Agricola R, Price A, Vincent T, Weinans H, Carr A. Osteoarthritis. Lancet 2015.

Goldring M, Marcu K. Cartilage homeostasis in health and rheumatic diseases. Arthritis Res Ther 2009; 11:224.

Henrotin Y, Priem F, Mobasheri A. Curcumin: a new paradigm and therapeutic opportunity for the treatment of osteoarthritis: curcumin for osteoarthritis management. Springerplus 2013; 2:56.

Hu C, Liu J, Cheah K, Li J, Lam C, Yu W, Choy C. Heme oxygenase-1 mediates the inhibitory actions of brazilin in RAW264.7 macrophages stimulated with lipopolysaccharide. J Ethnopharmacol 2009; 121:79-85.

Jung E, Han K, Hwang S, Kwon H, Patnaik B, Kim Y, Han M. Brazilin isolated from Caesalpinia sappan L. inhibits rheumatoid arthritis activity in a type-II collagen induced arthritis mouse model. BMC Complement Altern Med 2015; 15:124.

Jung Y, Kim I. Total synthesis of brazilin. J Org Chem 2015; 80(3):2001-5.

Kim N, Mehta R, Yu W, Neeman I, Livney T, Amichay A, Poirier D, Nicholls P, Kirby A, Jiang W, Mansel R, Ramachandran C, Rabi T, Kaplan B, Lansky E. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Cancer Res Treat 2002; 71:203–217.

Lee Y, Noh E, Han J, Kim J, Hwang J, Hwang B, Chung E, Kim B, Lee SH, Lee SJ, Kim J. Brazilin inhibits UVB-induced MMP-1/3 expressions and secretions by suppressing the NF-κB pathway in human dermal fibroblasts. Eur J Pharmacol 2012; 674: 80-86.

Lefèvre S, Knedla A, Tennie C, Kampmann A, Wunrau C, Dinser R, Korb A, Schnäker E, Tarner I, Robbins P, Evans C, Stürz H, Steinmeyer J, Gay S, Schölmerich J, Pap T, Müller-Ladner U, Neumann E. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med 2009; 15:1414-20.

Liu L, Gu H, Liu H, Jiao Y, Li K, Zhao Y, An L, Yang J. Protective effect of resveratrol against IL-1β-induced inflammatory response on human osteoarthritic chondrocytes partly via the TLR4/MyD88/NF-κB signaling pathway: an "in vitro study". Int J Mol Sci 2014; 15:6925-40.

Madry H, van Dijk C, Mueller-Gerbl M. The basic science of the subchondral bone. Knee Surg Sports Traumatol Arthrosoc 2010; 18:419-33.

Magdalou J, Chen L, Wang H, Qin J, Wen Y, Li X, Shang L, Li J. Angelica sinensis and osteoarthritis: a natural therapeutic link? Biomed Mater Eng 2015; 25:179-86.

Pearle A, Warren R, Rodeo S. Basic science of articular cartilage and osteoarthritis. Clin Sports Medicine 2005; 24:1-12.

Pelletier J, Yaron M, Haraoui B, Cohen P, Nahir M, Choquette D, Wigler I, Rosner I, Beaulieu A. Efficacy and safety of diacerein in osteoarthritis of the knee: a double-blind, placebo-controlled trial. The Diacerein Study Group. Arthritis Rheum 2000; 43:2339-48.

Salesi M, Meidani M, Meshkinfar S, Hashemi H, Farajzadegan Z. Purified protein

derivative test and its booster phenomenon in patients with rheumatoid arthritis. Adv Biomed Res 2015; 4:80.

Shukla M, Gupta K, Rasheed Z, Khan K, Haqqi T. Bioavailable constituents/ metabolites of pomegranate (Punica granatum L) preferentially inhibit COX2 activity ex vivo and IL-1beta-induced PGE_2 production in human chondrocytes in vitro. J Inflamm (Lond) 2008; 5: 9.

Smith M. The Normal Synovium. Open Rheumatol J 2011; 5:100-6.

Toegel S, Wu S, Otero M, Goldring M, Leelapornpisi P, Chiari C, Kolb A, Unger F, Windhager R, Virnstein H. Caesalpinia sappan extract inhibits IL-1β-mediated overexpression of matrix metalloproteinases in human chondrocytes. Gene Nutr 2012; 7:307-18.

Wang X, Zhang H, Yang X, Zhao J, Pan C. Enantioselective total synthesis of (+)-brazilin, (-)-brazilein and (+)-brazilide A. Chem Commun (Camb) 2013; 49(47):5405-7.

Wechalekar M, Smith M. Utility of arthroscopic guided synovial biopsy in understanding synovial tissue pathology in health and disease states. World J Orthop 2014; 5: 566-73.

Wu S, Otero M, Unger F, Goldring M, Phrutivorapongkul A, Chiari C, Kolb A, Virnstein H, Toegel S. Anti-inflammatory activity of an ethanolic Caesalpinia sappan extract in human chondrocytes and macrophages. J Ethnopharmacol 2011; 138:364-72.

6.3. Websites

http://de.slideshare.net/maeladl/anatomy-of-the-knee-joint-32431921

http://parisaramahiti.kar.nic.in/medicinial_plants_new/med%20plants/p26.html

http://www.arthroseorthopaedie.de/images/knieanatomie_1_l.gif)

http://www.med-library.com/synovia-gelenkfluessigkeit-knie-infos-gelenkschmiere-knie/

http://www.rheumatologynetwork.com/rheumatoid-arthritis/managing-ra-2011-update

http://www.yobo360.com/shop365/product-2359.aspx

7. Appendix

7.1. Abbreviations

C	control
CSE	ethanolic extract of Caesalpinia sappan
DMARDs	disease modifying anti-rheumatic drugs
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
F	fraction
FBS	fetal bovine serum
HMOX-1	heme Oxygenase 1 protein
HMOX1	heme Oxygenase 1 gene
HPLC	High Performance Liquid Chromatography
IL-1β	interleukin 1 beta protein
IL1B	interleukin 1 beta gene
MDB	membrane desalting buffer
MMP-13	matrix metalloproteinase 13 protein
MMP13	matrix metalloproteinase 13 gene
NSAIDs	non-steroidal anti-inflammatory drugs
NFκB	nuclear factor-κB
OA	osteoarthritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

RA	rheumatoid arthritis
rpm	revolutions per minute
RT-qPCR	quantitative real time PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	trifluor acetic acid
TNFα	tumor necrosis factor alpha

7.2. List of Figures

7.3. List of Tables

8. Curriculum vitae

Personal information

Name: Athena Su Agin

Date of birth: 21.11.1990

Education

1996-2000 Primary school: Private school, Schulbrüder De La Salle; Vienna

2000-2009 Secondary school: Private school, Schulschwestern Notre Dame; Vienna

8th June 2009 School leaving examination, Matura

2009-2015 Study of pharmacy, University of Vienna

Languages

German	Englisch	Greek	Turkish
First language	B2	B1	B1

Career related activities

2006 Personality and communication training

2007 Economic internship, SISLEY, Vienna

2008 Social internship, Caritas, archdiocese Vienna, house "St. Barbara"

2008 Intensive English language program in Cambridge, England

2005-2009 attendant to the secondary school, Greek school of Vienna

November 2010- March 2012 temporary employee at DELKA GmbH & Co KG

2015 temporary employee at Laaerberg Apotheke, 1100 Wien

Other: Private lessons at mathematic, physic, chemistry and English for students, babysitter

November 2014- June 2015 thesis research in: Karl Chiari Lab for Orthopaedic Biology, general hospital Vienna